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XVIII. THE REACTION AND CALCIUM CONTENT OF MILK AS FACTORS IN THE COAGULATION PROCESS.

By THOMAS HUGH MILROY.

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(*Received April 12th, 1915.*)

The two main factors which govern the coagulant action of rennin on caseinogen are the reaction and the calcium content of the medium. Within recent years the influence of variations in the reaction upon this process have been studied especially by van Dam [1908], Allemann [1912] and Michaelis and Mendelssohn [1913], all of whom have employed the electrometric method for the estimation of the actual acidity or hydrogen ion concentration, [H·][1]. Van Dam, who adopted a rather ingenious method of determining the coagulation time in specimens of milk which were only slightly diluted, came to the conclusion that the rate of coagulation was directly proportional to the [H·] for the comparatively limited range which he investigated. After studying the action of $CaCl_2$ upon the coagulation period, he came to the conclusion that in so far as this salt raised the [H·] of the mixture, it also hastened the formation of soluble casein from caseinogen. The specific action of the calcium was dependent not upon the calcium salts in solution in the milk, but upon the amount of calcium in colloidal combination. Allemann estimated the [H·] of milk after the addition of equivalent amounts of hydrochloric and acetic acids and found that the [H·] was the same for equivalent concentrations of the two acids although the degree of dissociation in the two cases was so different. This is evidently due to the fact that the milk acts as a reaction regulator from its alkaline phosphate content and the regulating power is lost when all the secondary is transformed into primary phosphate, acid precipitation of the caseinogen occurring at this point. The author also pointed out that prior to the precipitation of caseinogen by acid—stated quantitatively, with lower [H·] than $1{\cdot}25 \cdot 10^{-5}$ N

[1] [H·]=Hydrogen ion concentration.

$p_{H\cdot}$=The negative exponent of the same when the normality is expressed as a power of 10.

(from $0{\cdot}64 \cdot 10^{-5}$ to $1{\cdot}25 \cdot 10^{-5}$)—a zone of opalescence is met with and it is within this zone that the coagulant action of rennin takes place.

Michaelis and Mendelssohn investigated the subject in rather a different way from the others. They determined the optimum zones of $[H^{\cdot}]$ for the coagulant action of rennin and the precipitation of caseinogen by acid. They found the optimum zone for rennin action in the presence of soluble calcium salts to lie between $0{\cdot}4 \cdot 10^{-6}$ and $1 \cdot 10^{-6}$ N. It was not possible to narrow this zone down further, but in their opinion $1 \cdot 10^{-7}$ and $3 \cdot 10^{-6}$ lay outside the region of optimal activity. The optimum zone for acid precipitation of caseinogen in pure solution or in milk was found to lie about $2{\cdot}5 \cdot 10^{-5}$ N, while in $m/40$ $CaCl_2$ solution optimum precipitation was obtained with higher $[H^{\cdot}]$, namely about $3 \cdot 10^{-4}$ N. The authors therefore state that there is a region between 10^{-6} and 10^{-5} in which neither optimal rennin action nor optimal acid precipitation takes place. They therefore do not regard the zone of rennin action as simply an extension of the acid precipitation zone. Making use of acetate regulator mixtures to eliminate the rise in $[H^{\cdot}]$ produced by the addition of $CaCl_2$, they came to the conclusion that the $Ca^{\cdot\cdot}$ action in rennin coagulation is not simply an expression of the $[H^{\cdot}]$ rise. In studying the parts played by variations in $[H^{\cdot}]$ and in the soluble calcium content on the coagulation process, it is essential to devise experimental conditions which render it possible to effect a change in the one without at the same time altering the other. It is necessary therefore to avoid the $[H^{\cdot}]$ variations produced by the addition of calcium chloride to milk, and hence the use of regulator mixtures is required. In the investigation however of the rennin coagulation process, it is also necessary to determine whether during the course of enzyme action there is any change in $[H^{\cdot}]$ such as occurs, for example, in the case of peptic digestion [Sörensen, 1908, 1912; Christiansen, 1912], in which at the outset the combining power of the digestion products for bases is increased and subsequently decreased. In the case of such an investigation, as also in the study of the effects produced on $[H^{\cdot}]$ by substances which accelerate or retard coagulation, it is necessary to avoid the use of reaction regulators.

At the outset therefore the $[H^{\cdot}]$ of fresh milk was determined before and during the course of digestion and without the addition of a reaction regulator mixture.

The method employed for the determination of the $[H^{\cdot}]$ was the electrometric one as described by Sörensen and others. I have given certain details as to my own method of procedure in another paper [1914]. It is quite

unnecessary to give a list of all the determinations of the [H˙] of fresh milk, but as the values depend partly upon the method employed for saturating the milk specimen in the electrode with hydrogen, it is necessary to refer briefly to the effect of bubbling the gas through the milk. When one employs the "stationary" hydrogen electrode (as used by Hasselbalch, Michaelis and others) the values are slightly higher than when the gas is bubbled through the milk, even when the precaution is taken of passing the gas through a series of wash bottles containing milk before it enters the electrode. The fall in [H˙] is evidently due to the removal of CO_2, but this can be greatly diminished by passing the gas through a number of milk tubes before it enters the electrode.

The [H˙] of Fresh Undiluted Milk.

From a very large number of specimens examined, the range of [H˙] in fresh milk was found to be from $0{\cdot}158 \,.\, 10^{-6}$ N (p_H. 6·80) to $0{\cdot}310 \,.\, 10^{-6}$ N (p_H. 6·50) as determined by the stationary hydrogen electrode method. When hydrogen is bubbled through the milk in the electrode, with the precautions taken which have just been mentioned, the [H˙] range is from $0{\cdot}140 \,.\, 10^{-6}$ to $0{\cdot}158 \,.\, 10^{-6}$ N. The great majority of the fresh milk specimens have a [H˙] from $0{\cdot}18 \,.\, 10^{-6}$ (p_H. 6·75) to $0{\cdot}25 \,.\, 10^{-6}$ (p_H. 6·60) as determined by the stationary electrode method.

The [H˙] during the Coagulation Process.

This is most easily estimated either in milk which has been diluted or in milk which has been first rendered non-coagulable by the addition of alkaline oxalate. The coagulation process can be readily followed in the oxalated milk on the addition of $CaCl_2$. The addition of potassium oxalate to milk causes a fall in [H˙] owing to the hydrolytic dissociation of the salt.

Thus 1 cc. $K_2C_2O_4 \,.\, 4H_2O$ (3 %) added to 10 cc. milk—i.e. about $m/80$ oxalate concentration—gives rise to a fall in [H˙] from $0{\cdot}19 \,.\, 10^{-6}$ N to $0{\cdot}06 \,.\, 10^{-6}$ (p_H. 6·70 to 7·22). That is to say the addition of a sufficient quantity of potassium oxalate to render milk non-coagulable lowers the [H˙] to such an extent that the change in reaction alone, apart altogether from the calcium precipitation, would place the milk outside the range of Michaelis' coagulation zone. One must therefore bear in mind that the depression of coagulability produced by the addition of oxalate must be in part due to the lowered [H˙].

If rennin be added to an "oxalate" milk and the mixture kept in a water-bath at 38° the [H˙] is not found to change during the period of digestion.

An example may be given of such a milk specimen which remained unclotted throughout the experiment.

TABLE I.

					$[H^{\cdot}] \times 10^8$ N.	$p_{H^{\cdot}}$
Fresh milk					0·477	6·70
					$[H^{\cdot}] \times 10^7$	$p_{H^{\cdot}}$
Same containing $m/80$ $K_2C_2O_4$, after 10 min. digestion					0·707	7·15
,,	,,	,,	20	,,	0·724	7·14
,,	,,	,,	30	,,	0·724	7·14
,,	,,	,,	60	,,	0·741	7·13

When $CaCl_2$ is added to an "oxalate" milk the $[H^{\cdot}]$ rises, but the milk remains uncoagulable until the $[H^{\cdot}]$ reaches a higher level than p_H. 6·86. The following examples of different specimens of milk are given in order to show the effects produced by oxalate and $CaCl_2$ on the $[H^{\cdot}]$ of milk subjected throughout to the action of rennin at 38°.

The E.M.F. of the fluid is given at different periods and the calculated $p_{H^{\cdot}}$ in order that any change in the $[H^{\cdot}]$ throughout the course of the experiment may be observed.

TABLE II.

(1) Milk 10 cc., $K_2C_2O_4$ (3 %) 1 cc., Rennin (0·25 %) 1 cc. at 38°.

Period of digestion in minutes	E.M.F.	$p_{H^{\cdot}}$	Remarks
5	0·7552	7·23	fluid
10	0·7546	7·22	,,
15	0·7544	7·22	,,
	added 1 cc. $CaCl_2$ (1 %)		
	0·6962	6·21	curdling
6	0·6948	6·18	clotted
10	0·6947	6·18	,,
20	0·6949	6·18	,,

(2) Another specimen of milk containing the same amount of oxalate and rennin at 38°.

Period of digestion in minutes	E.M.F.	$p_{H^{\cdot}}$	Remarks
1	0·7508	7·15	fluid
5	0·7500	7·14	,,
10	0·7496	7·13	,,
15	0·7494	7·13	,,
	added 0·75 cc. $CaCl_2$ (1 %)		
1	0·7103	6·46	fluid
2	0·7110	6·47	,,
5	0·7127	6·49	curdling
10	0·7135	6·50	clotted
15	0·7135	6·50	,,
30	0·7135	6·50	,,

(3) Another specimen of oxalate milk and rennin at 38°.

Period of digestion in minutes	E.M.F.	$p_{H^{\cdot}}$	Remarks
1	0·7420	7·00	fluid
5	0·7400	6·97	,,
10	0·7391	6·97	,,
	added 0·3 cc. $CaCl_2$		
1	0·7343	6·87	fluid
3	0·7340	6·86	,,
6	0·7335	6·86	,,
10	0·7338	6·86	,,
	again added 0·3 cc. $CaCl_2$		
10	0·7166	6·56	clotted
20	0·7141	6·52	,,
25	0·7140	6·52	,,

Other specimens gave similar results. It is evident in the first place that the oxalate lowers the [H·] while the $CaCl_2$ raises it and that coagulation occurs when the [H·] reaches the level of that possessed by a normal coagulable milk.

During the period, however, when the ferment is acting on milk either containing an excess of oxalate or of $CaCl_2$ there is no definite change of reaction.

It is certain that in specimen (3) after the addition of 0·3 cc. $CaCl_2$, when the [H·] has been raised approximately to the lowest level of the optimum coagulation zone, viz. $p_{H^{\cdot}}$ 6·86–6·87, ferment action must have been proceeding during the ten minutes in which the potential estimations were made and yet there was no appreciable alteration in the E.M.F.

Therefore during the early period of the rennin action prior to the separation of a clot there is no indication of accompanying [H·] alterations such as are evident in pepsin action. Again, in the actual passage from the fluid to the clotted state there is also no definite indication of an alteration in the [H·] such as might occur from the fixation of calcium by the soluble casein giving rise to an increase in the [H·] of the fluid.

Effect of Variations in the Calcium Content and the Reaction of Milk on Coagulability.

As alkaline oxalate in addition to its effect upon the soluble calcium content of the milk also lowers the [H·], it was necessary either to counteract this effect upon the [H·] as, for example, by the addition of oxalic acid, or to adopt some other method of diminishing the soluble calcium content. It has been for a long time well known that milk which has been kept at a

temperature over 80° C. is gradually rendered less and less coagulable by rennin. Otto de Vries and Boekhout [1901] stated that the calcium content of such poorly coagulating milk is not lower than that of normal milk, although they granted that the addition of $CaCl_2$ rendered the milk coagulable. This was not due, however, in their opinion to an increased soluble calcium content of the milk, and they therefore concluded that the rise in acidity was the governing factor. Van Dam estimated the total calcium content of milk of normal coagulability and that of milk which either did not coagulate at all or only after a longer period than normal, and he found that the former contained about 0·154–0·174 % CaO, while the latter contained 0·135–0·150 % CaO. Klein and Kirsten [1901] state that heated milk on addition of $CaCl_2$ clots more readily with rennin than unheated milk.

Milk rendered uncoagulable by heating.

The milk was kept at a temperature slightly below boiling point for one hour, by placing it in the inner vessel of a double saucepan, the outer one containing boiling water. Such milk, which shows greatly reduced coagulability, was made use of in the experiments which follow. After the milk had been heated for one hour it was cooled, made up to the original volume and filtered after again being warmed. The first point to determine was the [H·] of the heated milk as compared with that of the same milk prior to heating. So far as I know, the [H·] of heated milk has not hitherto been determined.

The following table gives the [H·] of different specimens of milk before and after heating.

TABLE III.

[H·] *of Milk before and after heating.*

	Before			After		
	E.M.F.	$[H^{\cdot}] \times 10^7$ N	$p_{H^{\cdot}}$	E.M.F.	$[H^{\cdot}] \times 10^7$ N	$p_{H^{\cdot}}$
(1)	0·7174	2·63	6·58	0·7126	3·25	6·49
(2)	0·7254	1·90	6·72	0·7162	2·81	6·55
(3)	0·7274	1·77	6·75	0·7174	2·63	6·58
(4)	0·7273	1·77	6·75	0·7190	2·51	6·60
(5)	0·7304	1·58	6·80	0·7190	2·51	6·60
(6)	0·7310	1·55	6·81	0·7250	1·95	6·71

In every case the heated milk shows a higher [H·] than the same milk before heating.

The [H·] of heated milk specimens remains also much more constant than that of fresh milk. Heating therefore produces exactly the opposite effect on the [H·] from that produced by the addition of potassium oxalate.

In order to understand the cause of this rise in acidity attention requires to be directed to the calcium content of the milk. The percentage of CaO in different specimens of milk before and after heating is shown in the following table.

TABLE IV.

Percentage of CaO *in Milk before and after heating.*

	Before CaO %	After CaO %
(1)	0·1738	0·1345
(2)	0·1738	0·1524
(3)	0·1630	0·1416
(4)	0·1577	0·1437
(5)	0·1542	0·1447

These results show an average of 0·1645 % CaO in unheated and 0·1434 % CaO in heated milk. The rise in [H·] which occurs on heating milk is evidently due therefore to the separation of calcium phosphate in more basic form than it previously existed in the milk, the transformation probably being of the following nature:

$$3CaHPO_4 + Na_2HPO_4 = Ca_3(PO_4)_2 + 2NaH_2PO_4.$$

The removal of carbonic acid from the milk by heating lowers the solubility of the calcium phosphates.

According to Conradi [1901] the diminished coagulability of heated milk with rennin goes hand in hand with an increase in coagulability on heating with calcium chloride. This is undoubtedly the case, for if one takes two 5 cc. specimens of fresh and heated milk, one finds that after adding 0·5 to 0·6 cc. $CaCl_2$ (1 %) to each, the heated specimen coagulates on boiling, while the fresh one does not coagulate until 0·8–0·9 cc. is added.

Action of rennin on heated milk.

As above stated, the coagulant action of the ferment on heated milk is very much diminished and that this decrease in activity is not due to the alteration in reaction is evident from the fact that the increase in [H·] produced by heating milk ought of itself to increase the coagulability. That the diminished coagulability is due to the lowered calcium content of the milk is evident from the fact that on raising the percentage of calcium in heated milk to the same level as in the milk before heating, the milk coagulates as readily as the fresh milk. As already stated, however, the addition of calcium chloride to the milk raises the [H·] unless the milk be diluted with some reaction regulator such as the acetate mixture employed by Michaelis.

When heated milk is diluted with sodium acetate ($m/4$) the [H·] is lowered owing to the hydrolytic dissociation of the alkaline acetate. On adding $CaCl_2$ to this mixture the [H·] at first rises owing to the lower dissociation constant of the calcium acetate; with further additions of $CaCl_2$, however, the [H·] only alters slightly. Thus, for example, the $p_{H·}$ values of certain acetate and acetate-$CaCl_2$ milk mixtures are as follows:

	$p_{H·}$
(1) Heated milk (not diluted)	6·56
(2) Heated in $m/4$ sodium acetate (milk 1 in 4 dilution)	6·97
(3) Same as (2) but containing $m/200$ $CaCl_2$	6·81
(4) Same as (2) but containing $m/40$ $CaCl_2$	6·73

In the subsequent experiments N sodium acetate was added to the milk.

Addition of $CaCl_2$ *to heated milk.*

The following series illustrates the action of $CaCl_2$ on the coagulability of heated milk diluted as follows,

Milk	10 cc.
Sodium acetate N ..	2 cc.
Water to	40 cc.

To this mixture various amounts of $CaCl_2$ were added and the [H·] and coagulation time noted.

TABLE V.

1 %$CaCl_2$	E.M.F.	[H·] × 10^6 N	$p_{H·}$	Coagulation time in minutes
2 cc.	0·7307	0·15	6·81	6
4	0·7257	0·19	6·72	5
6	0·7207	0·23	6·63	3
8	0·7198	0·24	6·62	2·5
10	0·7195	0·24	6·61	2

Although it is evident that the addition of the $CaCl_2$ solution has produced a rise in [H·], this is inappreciable after the initial increase. The shortening in the coagulation period to one-third of the original has taken place with an 80 % increase in the [H·]. In many cases the rise in [H·] produced by the addition of $CaCl_2$ is even less marked, as in the specimens previously referred to where a $m/200$ $CaCl_2$ in milk-sodium acetate had a $p_{H·}$ of 6·81, while a $m/40$ $CaCl_2$ showed a rise in [H·] only to $p_{H·}$ 6·73. Yet in this case the shortening in the coagulation time was approximately the same as in the last mentioned series.

The factor concerned in the shortening of the coagulation period in this series has evidently not been the slight rise in [H·] because such a rise

otherwise produced than by addition of $CaCl_2$ would scarcely have shortened the coagulation period.

If we now determine the rise in [H˙] which is required in order to render heated milk coagulable without the further addition of $CaCl_2$ we obtain results which make it evident that the calcium chloride does not produce its effect simply from the rise in [H˙] which it produces.

The coagulation zone of heated milk diluted with sodium acetate and acetic acid.

Heated milk may be again rendered coagulable by rennin not only by an increase in the soluble calcium content without an appreciable rise in the [H˙], but also by a rise in the [H˙] without an increase in the soluble calcium content except in so far as the increasing acidity may produce a slight effect.

When heated milk is diluted with sodium acetate, the [H˙] falls. This fall is prevented by the addition of acetic acid. The following table gives the [H˙] and observations on the coagulation of milk-acetate-acetic acid mixtures of different [H˙].

TABLE VI.

Milk 10 cc., Sodium acetate (N) 10 cc., Rennin (0·25 %) 2 cc., Water up to 40 cc.

	E.M.F.	$[H^{\cdot}] \times 10^7$ N	$p_{H^{\cdot}}$	
Without acid	0·7416	1	7·00	no clot
0·6 cc. acetic acid (N)	0·6920	7·24	6·14	,,
1·2 ,, ,,	0·6717	16·69	5·78	clotted
1·5 ,, ,,	0·6661	20·42	5·69	,,
2 ,, ,,	0·6577	28·84	5·54	,,

From a comparison of Tables V and VI it is evident that coagulation takes place at a much lower [H˙] when $CaCl_2$ is added to the heated milk than when acetic acid is added, namely in the case of the former with [H˙] from 0·15 to 0·24 $\times 10^{-6}$ N, in the latter on the other hand only when the [H˙] is higher than 0·724 $\times 10^{-6}$. There are therefore two variables to be considered in the case of the rennin action on caseinogen, namely the [H˙] of the fluid and its calcium content.

Before discussing the relationship of these variables to one another, it is advisable to refer briefly to the acid precipitation zone of caseinogen in the absence of ferment, in order to see whether the ferment zone is not after all simply an extension of the acid precipitation zone.

If heated milk be diluted with sodium acetate-acetic acid mixture as in the preceding series and the [H˙] determined at the point when caseinogen

is precipitated from the milk, one obtains results which show that the precipitation zone lies on the acid side of the coagulation zone. The milk-acetate-acetic acid mixtures were kept at 38°.

Milk	10 cc.
Sodium acetate (N)	10 cc.
Acetic acid N from	1 cc. upwards
Water to	40 cc. at 38°

Until the [H·] reached $0{\cdot}95 \cdot 10^{-5}$ there was no sign of a precipitate. The figures below give the acidities at which precipitation takes place.

E.M.F.	$[H^{\cdot}] \times 10^5$ N	$p_{H^{\cdot}}$	Remarks
0·6377	0·630	5·20	No ppt. in 1 hour
0·6275	0·955	5·02	ppt. in 6 min.
0·6236	1·122	4·95	ppt. in 4 min.

On repeating this experiment but with 2 cc. $CaCl_2$ (1 %) added the acid precipitation of caseinogen took place at practically the same [H·] as in the absence of the $CaCl_2$. A precipitate was obtained in 6 min. with a [H·] of $1{\cdot}04 \cdot 10^{-5}$ (p_H. 4·98).

Michaelis and his fellow workers give, as the most favourable [H·] for the precipitation of caseinogen in the absence of $CaCl_2$, $2{\cdot}5 \cdot 10^{-5}$ N (p_H. 4·60), which is a more acid reaction than the one I have just given. They employed, however, caseinogen solutions, and the conditions as regards temperature of mixture and sodium acetate concentration were different in the two cases.

Michaelis and Mendelssohn found that in the presence of $CaCl_2$ (in greater concentrations than those used by me) the acid precipitation zone of caseinogen is shifted still more to the acid side.

Although there is no doubt that the acid precipitation zone of heated milk lies some distance on the acid side of the coagulation zone, it is certain that with still further lowering of the calcium content of the milk, the zone of rennin action would approach more nearly that of acid precipitation.

The [H·] of milk therefore requires to be highest for the precipitation of caseinogen by acid from solutions of its salts.

The separation of caseinogen from its solutions by the action of rennin takes place at lower [H·], the exact point at which coagulation takes place being dependent upon the soluble calcium content. It is highest when the calcium content is low and gradually falls as the latter is raised.

What then are the exact relations of these two variables to one another?

The value of the one variable [H·], which corresponds to a definite value of the other variable (calcium) for a fixed coagulation time, has been determined in a series of milk-acetate-acetic acid mixtures. The coagulation time

which was taken was 5 min., and the calcium content and [H·] of the various milk-acetate mixtures determined which showed coagulation by rennin within that time.

Beginning with a milk-acetate mixture made up as follows:

Heated milk	10 cc.
Sodium acetate (N)	10 cc.
Rennin (0·25 %)	2 cc.
Water to	40 cc.

the [H·] and CaO percentage of the diluted mixture were determined and found to be p_H. 7·00 and 0·035 % CaO respectively. This mixture did not clot.

To a series of milk-acetate mixtures of the above composition acetic acid (normal) was added (starting with 0·1 cc.) until one was obtained which clotted in five minutes.

It was found that a milk-acetate-acetic mixture of [H·] $0{\cdot}112 \cdot 10^{-5}$ clotted in ten minutes; one of $0{\cdot}157 \cdot 10^{-5}$ in seven minutes; and one of $0{\cdot}177 \cdot 10^{-5}$ in five minutes.

Thus a milk specimen of 0·035 % CaO required to have the [H·] raised to $0{\cdot}177 \cdot 10^{-5}$ to obtain coagulation in five minutes.

The calcium content of the diluted milk was then gradually increased by the addition of small quantities of $CaCl_2$ (1 %). After each addition of $CaCl_2$ a series of milk-acetate-acetic acid mixtures of the same calcium content but of different [H·] was prepared, and the [H·] of the one which showed coagulation in five minutes was accurately determined.

The following table gives the results:

	CaO %	[H·] × 10^6 N	p_H·
(1)	0·035	1·778	5·75
(2)	0·036	1·660	5·78
(3)	0·038	0·871	6·06
(4)	0·042	0·309	6·51
(5)	0·050	0·230	6·64
(6)	0·060	0·186	6·73
(7)	0·073	0·154	6·81
(8)	0·085	0·135	6·87
(9)	0·122	0·126	6·90
(10)	0·135	0·107	6·97

From this table it is evident that when the calcium content is brought below 0·06 % and especially when it has fallen to 0·042 % the [H·] requires to be proportionately much more increased in order to enable coagulation to take place within the five-minute period. Thus in the range from 0·060 to 0·035 % CaO, the [H·] requires to be increased tenfold. This range in

calcium content represents the difference between fresh milk and heated milk diluted to the same extent with the acetate mixture.

The specimens of heated milk diluted with the acetate mixtures which possess hydrogen ion concentrations corresponding to those of fresh coagulable milk have a CaO percentage similar to those of fresh coagulable milk diluted to the same extent, namely specimens (4), (5) and (6). In the specimens above (6) NaOH (N/10) was added in order to obtain the lower $[H^{\cdot}]$.

The Rôle of the Calcium Salts in the Formation of Soluble Casein.

In the preceding experiments attention has been merely directed to the separation of the casein clot, and practically no reference was directly made to the earlier part in the coagulation process, namely the formation of soluble casein. The part which the calcium content of the milk plays in this earlier process is difficult to dissociate from that played by the accompanying reaction changes.

The question to decide is whether the transformation of caseinogen to soluble casein takes place at the same rate in the case of a milk specimen of lowered calcium content as in one possessing a normal amount. Various methods were employed in order to decide this question. A few examples illustrating these methods may be given.

Two diluted milk specimens of the following composition were taken:

(1)	Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
	$CaCl_2$ (1 %)	2 cc.	
	Rennin (0·25 %)	2 cc.	
	Water to	40 cc.	

These specimens were kept at 38°. Clotting took place in specimen (1) in two minutes. Immediately on the appearance of the clot in this specimen, 2 cc. $CaCl_2$ (1 %) were added to the other specimen, which up to this time had shown no clot. Coagulation occurred two minutes later. On this being repeated with a smaller amount of rennin solution, coagulation occurred in specimen (1) in 3·5 minutes, and in specimen (2) 3·5 minutes after the addition of $CaCl_2$. That is to say in neither of these cases did the preliminary digestion in a diluted milk specimen without the addition of $CaCl_2$ shorten the subsequent period of coagulation after the addition of $CaCl_2$.

One must bear in mind, however, that the addition of $CaCl_2$ not only increases the calcium content of the milk but it also, especially in milk simply diluted with water, raises the $[H^{\cdot}]$, and thus specimen (1) was

placed under more favourable circumstances, as regards reaction, for the activity of the rennin. The addition of water alone lowers the [H·] and so specimen (2) was placed under more unsatisfactory conditions for the enzyme action.

It is necessary therefore to compare two diluted milk (heated) specimens of approximately the same [H·], the one containing $CaCl_2$, the other without this addition as was done in the following experiment:

(1)	Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
	Sodium acetate (N)	10 cc.	
	Acetic acid (N/10)	0·5 cc.	
	$CaCl_2$ (1 %)	1 cc.	
	Rennin (0·25 %)	2 cc.	
	Water to	40 cc.	

Both specimens had a [H·] of approximately $2 \cdot 10^{-7}$ N. Specimen (1) clotted in 5·75 min. Immediately on appearance of coagulation in this specimen 1 cc. $CaCl_2$ (1 %) was added to the other, which at this time showed no sign of coagulation. Coagulation occurred in this second specimen 2·75 min. later. That is to say the preliminary digestion with rennin in a milk-acetate mixture to which no $CaCl_2$ was added has shortened the period of coagulation after the addition of $CaCl_2$ by rather more than one half. Evidently therefore, although as was to be expected the transformation of caseinogen into soluble casein takes place in such a mixture when no $CaCl_2$ has been added, the rate of transformation is slower than in the presence of a small quantity of that salt, even when the [H·] in both cases is the same.

That the addition of $CaCl_2$ does not act simply as a precipitant of soluble casein is shown also from the following experiment.

20 cc. heated milk were taken, diluted 1 in 4 with water and kept at 38°. The mixture was divided into three portions. One of these was poured into a flask containing 2 cc. rennin and 1 cc. $CaCl_2$ (1 %), the other two portions into flasks containing 2 cc. rennin alone, and all were kept at 38°. The first specimen clotted in four minutes. One of the remaining specimens was then brought to boiling point, in order to destroy the rennin, and then cooled to 38°, while the third specimen was kept at 38°. When 1 cc. $CaCl_2$ (1 %) was added to each of these specimens after four minutes, the one in which the rennin had been destroyed did not clot, while the third specimen clotted in rather more than one minute.

In both of these specimens during the four minutes' action of rennin the caseinogen must have been acted upon by the ferment to such an extent that on the addition of 1 cc. $CaCl_2$ coagulation would take place after the

lapse of little over a minute, but this occurred only in the case where the ferment was allowed to continue its action during that extra period.

It is evident therefore that the action of the soluble calcium salt is not simply to complete a process the earlier stages of which do not require its presence.

From the beginning of the ferment action until its close the rate of transformation of caseinogen into the final product of digestion is dependent upon the calcium content and reaction of the medium.

Conclusions.

1. During the course of rennin action there is no change in the [H˙] of milk, either in the earlier stage or in the actual separation of the clot.

2. The addition of an alkaline oxalate to milk lowers, while that of $CaCl_2$ raises, the [H˙].

3. Fresh milk which has been subjected to a temperature slightly below boiling point for one hour shows a rise in [H˙], and a fall in the calcium content. Such milk is only very slowly acted upon by rennin.

4. The coagulability of heated milk may be raised either by the addition of $CaCl_2$ or by raising the [H˙]. The former does not act simply by raising the [H˙] nor the latter from its effect upon the soluble calcium content.

5. The acid precipitation zone of caseinogen lies on the acid side of the rennin zone of action, but the latter gradually approaches the former as the calcium content of the mixture is lowered, so that in all probability the latter is an extension of the former towards the neutral point.

6. Calcium chloride, apart from its effect upon [H˙], increases the activity of the rennin ferment from the beginning of the digestion process.

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XIX. THE RELATIONSHIP OF CREATINURIA TO CHANGES IN THE SUGAR CONTENT OF THE BLOOD.

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Previous Work and Object of present Research.

An increase in the normal sugar content of the blood has long been known to be an associated phenomenon of certain pathological conditions, especially pancreatic diabetes or the glycosuria artificially produced by the injection of certain drugs, e.g. uranium. Of recent years it has been shown that a diminution in the normal sugar content, or a hypoglycaemia, may follow the injection of certain other drugs, which cause considerable hepatic disturbance accompanied by the excretion of creatine. Some previous work by the writer [MacAdam, 1913] in corroboration of the hypothesis put forward by Cathcart [1909] that carbohydrates are intimately associated with the metabolism of creatine may explain the onset of this creatinuria.

Frank and Isaac [1911] by the administration of phosphorus to rabbits produced a distinct hypoglycaemia along with marked liver necrosis. The injection of hydrazine sulphate, which has a specific action in causing hepatic necrosis [Wells, 1908], leads to changes in the percentage of sugar in the blood, as shown by Underhill [1911; Underhill and Fine, 1911] both in dogs and rabbits. As the result of numerous experiments on dogs he found that a dose of 50 mgrms. per kilo. invariably produced an appreciable hypoglycaemia, although considerable variations in the degree of diminution of the sugar content occurred. His experiments with rabbits were not so conclusive. On the administration of hydrazine in doses of 50 mgrms. per kilo. a constant decrease in the sugar content was not obtained; for, although a hypoglycaemia of varying amount was found to occur in the

majority of the experiments, in others the blood sugar remained unaltered. On the administration of 100 mgrms. per kilo. the animals died within 24 hours, and Underhill concludes "From this diversity of results it is obvious that the rabbit cannot be relied on invariably to exhibit hypoglycaemia after hydrazine injection."

In these experiments the relationship of hypoglycaemia to creatinuria was not considered, and no attempt was made by the administration of intermediate doses to allow for the idiosyncrasy of individual rabbits. The object of the following experiments has been to try to determine experimentally whether this irregular occurrence of hypoglycaemia in rabbits depends upon the dose of hydrazine injected, and if it is in any way associated with the simultaneous occurrence of creatine in the urine.

Experiments and Methods of Analysis.

The rabbit was kept in a small cage over a funnel leading to a vessel in which the urine was collected. 5 cc. of 5 % H_2SO_4 were placed in this vessel previous to collection to prevent alkaline changes in the urine. At first a difficulty was experienced in obtaining the urine creatine-free during the control days. By means of a carbohydrate-rich diet this was in most instances surmounted or at least so diminished that the amount of creatine still present was negligible. In a few cases persistent creatinuria was found to be dependent upon pregnancy, and the rabbits were accordingly discarded. No attempt was made to obtain the quantitative amounts of creatine and creatinine excreted, partly on account of unequal quantities of urine being passed during each 24 hours, partly because such absolute figures have no direct bearing on the object of the present research. *Percentage* results are dealt with in Table I, the relationship of the excretion of creatine to total creatinine being thereby shown.

Control estimations of the sugar content were first made from 7–10 days previous to the injection of a solution of hydrazine sulphate (2·5 %). This was done without doing any injury to the vascular system of the animal, for after some practice no difficulty was experienced in obtaining from 20–30 cc. of blood from the auricular vein. The blood was dropped directly into a 50 cc. flask containing 5 cc. of a 2 % solution of potassium oxalate, and by the addition of the requisite amount of distilled water from a burette to bring the total amount of fluid up to 50 cc., the quantity of blood obtained was accurately determined. In two of the experiments recorded where the rabbits after the administration of hydrazine became comatose, it was found

impossible to obtain blood from the auricular vein, and hence it was drawn directly from the jugular vein and heart. The sugar content was estimated by the method of Allihn in terms of CuO.

The accuracy of the technique was tested in the case of a dog used for the purpose of another research [MacAdam, 1913] which became comatose within 24 hours after the injection of 90 mgrms. per kilo. of hydrazine. The dog was killed and from its jugular vein two quantities of blood, 32·2 cc. and 60 cc., were obtained, the sugar content of which was found to be 0·064 % and 0·068 % respectively. The sugar content of the normal dog is constant within narrow limits and averages 0·1 %, so that it is interesting to note that the above estimations corroborate the work of Underhill [1911], who found a sugar content of 0·05 % in the blood of a dog one day after the injection of 100 mgrms. hydrazine per kilo. of body weight.

In the initial experiments, doses of 50 mgrms. per kilo. body weight were administered, and 48 hours later blood was withdrawn, as above mentioned, for sugar estimation, while daily estimations of the urinary excretion of preformed creatinine and creatine were carried out.

If no symptoms were exhibited, and no creatinuria resulted, the same rabbit was injected again but with a larger dose in order to try just to exceed the tolerance of the animal's tissues to the poison. The varying doses used in each experiment are recorded in the protocols.

Since the animals, which showed metabolic disturbance, refused all food after the injection, two experiments (X and XI) with the rabbits fasting for two days were carried out in order to eliminate the factor of starvation from the results.

TABLE I.

(*The days of the control period in each experiment during which an attempt was being made to obtain the urine creatine-free are indicated by Arabic numerals, while the Roman figures refer to the days following the injection of the hydrazine.*)

I. *Rabbit A.* Weight 3·20 kilos.

Day	Total Creatinine (mgrms. in 100 cc. urine)	Preformed C. (mgrms. in 100 cc. urine)	Creatine as Creatinine (mgrms. in 100 cc. urine)	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
1	127·4	127·4	—	—	0·114	
2	101·5	98·3	3·2	3·1	—	
	Injection of 50 mgrms. Hydrazine sulph. per kilo.					
II	87·0	87·0	—	—	0·108	Animal showed no metabolic disturbance.

TABLE I (*continued*)

II. *Rabbit B.* Weight 2·73 kilos.

Day	Total Creatinine (mgrms. in 100 cc. urine)	Preformed C. (mgrms. in 100 cc. urine)	Creatine as Creatinine (mgrms. in 100 cc. urine)	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
1	71·0	65·5	5·5	7·4	0·095	
2	107·7	90·0	17·7	16·4	—	
3	—	—	—	—	—	
4	81·5	81·5	—	0·0	—	
	Injection of 50 mgrms. Hydrazine sulph. per kilo.					
I	72·5	74·1	—	—	—	Nothing abnormal was detected in the rabbit's condition after injection, food being taken as usual.
II	124·5	121·5	3·0	2·4	0·102	
III	110	115·0	—	—	—	

III. *Rabbit B.* Weight 2·47 kilos.

1	126·5	126·5	—	—	0·087	
2	107·5	98·5	9·0	8·3	—	
3	94·3	94·3	—	0·0	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	139·5	146·0	—	—	—	No metabolic disturbance noted.
II	124·5	126·5	—	—	0·107	
III	85·7	82·6	3·1	3·6	—	

IV. *Rabbit C.* Weight 1·77 kilos.

1	206·5	204·3	2·2	1·0	0·101	
2	87·3	87·3	—	—	—	
3	132·1	134·8	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	142·1[1]	135·0	7·1	5·0	—	Rabbit died within 12 hours of injection—no sugar estimation.

[1] Urine drawn from bladder post-mortem.

V. *Rabbit D.* Weight 2·32 kilos.

1	165·0	139·0	26·0	15·7	0·098	
2	—	—	—	—	—	
3	158·5	158·5	—	—	—	
4	72·0	73·5	—	—	—	
5	107·5	107·5	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
II	158·5	62·8	95·7	60·3	0·047	Rabbit showed considerable malaise, refusing all food after injection. Blood could not be obtained from auricular vein, and was taken from the jugular vein.

TABLE I (*continued*)

VI. *Rabbit A.* Weight 2·82 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	155·7	155·7	—	—	0·098	
2	128·4	125·2	3·2	2·4	—	
3	99·8	101·2	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
II	112·5	112·5	—	—	0·110	
III	154·0	156·5	—	—	—	No effects produced.

VII. *Rabbit A.* Weight 2·96 kilos.

1	171·0	162·0	9·0	5·2	—	
2	—	—	—	—	—	
3	62·5	64·5	—	—	—	
4	149·0	144·0	5·0	3·3	—	
	Injection of 100 mgrms. Hydrazine sulph. per kilo.					
II	217·0	135·0	82·0	37·8	0·150	The rabbit from the first day after injection showed considerable malaise. Blood was obtained with difficulty from the auricular vein, only 19·7 cc. being obtained.
III	279·0	208·0	71·0	25·4	—	

VIII. *Rabbit B.* Weight 2·87 kilos.

1	159·6	148·7	10·9	6·8	—	
2	—	—	—	—	—	
3	78·3	78·3	—	—	—	
4	122·1	122·1	—	—	—	
	Injection of 90 mgrms. Hydrazine sulph. per kilo.					
II	172·9	114·2	58·7	33·9	0·060	The usual symptoms of metabolic disturbance. Rabbit recovered, but was not used for further experiment.
III	96·3	68·4	27·9	20·9	—	

IX. *Rabbit F.* Weight 1·70 kilos.

1	137·3	137·3	—	—	0·09	
2	106·0	103·8	2·2	2·0	—	
3	143·4	145·2	—	0·0	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	43·6	39·1	4·5	10·3	0·094	Rabbit found comatose 24 hours after injection: 45 cc. of urine were removed from the bladder—acid in reaction. The blood was obtained directly from the heart.

TABLE I (*continued*)

X. *Rabbit G.* Weight 2·14 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	66·5	63·5	3·0	4·5	0·104	
2	130·0	130·0	—	—	—	
3	—	—	—	—	—	
4	106·5	102·6	3·9	3·6	—	
I	Fasting experiment					
II	187·4	143·5	43·9	23·4	0·140	After 2 days' starvation.
III	124·2	101·6	22·6	18·2	—	

XI. *Rabbit K.* Weight 1·91 kilos.

1	126·0	128·2	—	—	0·092	
2	137·0	137·0	—	—	—	
I	Fasting experiment					
II	132·5	95·7	36·8	27·7	0·116	After 2 days' starvation.
III	87·2	59·6	27·6	31·6	—	

XII. *Rabbit G.* Weight 2·0 kilos.

1	124·5	115·5	9·0	7·2	0·095	
2	97·4	99·2	—	—	—	
3	131·4	128·0	3·4	2·5	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
II	92·8	58·8	34·0	36·6	—	Rabbit showed usual symptoms of considerable malaise.
III	128·5	95·0	33·5	26·1	0·071	

XIII. *Rabbit M.* Weight 2·54 kilos.

4	102·5	74·3	28·2	27·5	0·105	
5	144·5	135·0	9·5	6·5	—	
6	58·6	54·3	4·3	7·3	—	
	Injection of 50 mgrms. Hydrazine sulph. per kilo.					
II	108·0	84·5	23·5	21·7	0·114	Animal aborted after withdrawal of blood.

XIV. *Rabbit K.* Weight 1·7 kilos.

1	—	—	—	—	0·101	
2	134·0	136·0	—	—	—	
3	98·4	98·4	—	—	—	
	Injection of 90 mgrms. Hydrazine sulph. per kilo.					
II	89·4	56·4	33·0	37·0	0·042	Rabbit was found comatose on morning of 2nd day, and 18·3 cc. of blood removed from the jugular vein. Urine was obtained directly from the bladder—acid in reaction

Summary of Results.

In the four experiments (I, II, III, VI) where the hydrazine administered was insufficient to produce any obvious metabolic disturbance no creatine was excreted, while the sugar content of the blood, as compared with the control estimation previously made, was within normal limits, when due allowance has been made for experimental error.

Of the six experiments, where creatinuria followed hydrazine injection, four (V, VIII, XII, XIV) showed a condition of hypoglycaemia; one (VII) a state of hyperglycaemia, while in the remaining one (IX) the sugar content remained unchanged. In this last experiment it may be noted that the sugar estimation was carried out on blood obtained directly from the heart 24 hours after the administration of the hydrazine; while the percentage $\frac{\text{creatine}}{\text{total creatinine}}$ was small, viz. 10%, when compared with the figures obtained in the "hypoglycaemia" experiments. Indeed it was not much higher than the percentage occasionally recorded during the control days.

In Experiment XIII, where the rabbit aborted after the withdrawal of the second quantity of blood, and where pregnancy had not been previously noticed, there is little difference between the two estimations although there is a distinct increase in the creatine percentage of the total creatinine.

In the two fasting experiments, the rabbit of Experiment X showed quite a marked hyperglycaemia, while in XI the sugar content of the blood showed a slight increase when compared with the control estimation. In both a marked creatinuria resulted.

In the remaining experiment (IV) Rabbit C died within 12 hours. No blood was obtained, while the urine drawn from the bladder post-mortem showed a trace of creatine. This was the only rabbit which exhibited such toxic symptoms after a dose of 75 mgrms. per kilo.

While this work was in progress Graham and Poulton [1913] published a communication in which it was suggested that an important fallacy underlies the supposed creatinuria occurring in diabetes and in conditions associated with acidosis, owing to the presence of aceto-acetic acid which tends to lower the value of the preformed creatinine estimations. In order to eliminate this error in the foregoing results, several experiments were performed with special reference to the possible presence of aceto-acetic acid. A positive result was never obtained with the ferric chloride test either before or after the injection of hydrazine. Owing to the extremely unsatisfactory nature of this test in the detection of minute amounts of this acid, if any suspicious

coloration was given with the ferric chloride solution the method recommended by Graham and Poulton was used. No trace of aceto-acetic acid was detected in any of the urines, while creatine was still found to be present in measureable amounts.

Discussion of Results.

The four experiments, in which no alteration in the sugar content of the blood, and no creatinuria, followed the injection of hydrazine, are interesting in relation to the previous work carried out by Underhill [1911].

In the present series of experiments the control estimations, carried out previous to the administration of hydrazine, show a reasonably constant sugar content of the blood, the average being approximately 0·10 per cent., thus corroborating previous investigations; while the divergence in the sugar estimations after injection appears to be dependent on the differences in susceptibility of the rabbits to the amount of the drug injected. Thus Rabbit A was used in Experiments I and VI, and Rabbit B in II and III, when 50 and 75 mgrms. per kilo. body weight were respectively administered with no resulting abnormal symptoms or creatinuria, while a dose of 75 mgrms. per kilo. had such a remarkably toxic effect on Rabbit C that it died within 12 hours of injection. After an interval of approximately one month, a larger dose was administered, 100 mgrms. per kilo. to Rabbit A (Experiment VII) and 90 mgrms. per kilo. to Rabbit B (Experiment VIII), with the result that both showed a distinct alteration in the percentage of their blood sugar, associated with the production of creatinuria. In all Underhill's experiments in which the blood sugar content was estimated, only 50 mgrms. per kilo. were administered, a dose which, in the case of the rabbits used in the present research, was never found to produce any alteration in sugar percentage. In the two other experiments he records, in which a dose of 100 mgrms. per kilo. was used, the rabbits died within five and twenty-four hours respectively, no sugar estimation being carried out. Hence it would appear that the rabbit can be relied upon to exhibit a constant hypoglycaemia after the administration of the amount of hydrazine requisite for each individual animal.

In the six experiments in which distinct creatinuria followed hydrazine injection, and in which no obvious extraneous factors could be detected, the sugar estimations show evidence of a well-marked associated hypoglycaemia. If the relationship of creatine to the total creatinine is considered, it is seen that after hydrazine injection the lowest percentage of creatine is 20·9,

the highest being 60·3 %, whereas during the three control days previous to injection the highest figure recorded, even in the case of those rabbits which were not obtained creatine-free, is 7·2 %. The only exception was Experiment II, in which on the third day previous to injection a percentage of 16·4 was recorded. However, before administration of the drug, no creatine was obtained, while after a dose of 50 mgrms. per kilo. the relationship of creatine to total creatinine remained within what were found to be normal limits. In the two fasting experiments the $\frac{\text{creatine}}{\text{total creatinine}}$ ratio ranged from 18·2 % to 31·6 %—figures quite in accordance with previous results.

As regards the sugar content of the blood in those four experiments where the occurrence of hypoglycaemia and creatinuria were associated, before injection the figures ranged from 0·087 %–0·101 %, while after injection they lay between 0·042 % and 0·071 %, there being thus always a distinct decrease in sugar percentage beyond the limits of experimental error.

Of the two experiments in which creatinuria was not associated with the simultaneous occurrence of hypoglycaemia, no appreciable alteration in the sugar percentage figure was found in Experiment IX; while in Experiment VII, Rabbit A, in which two months previously no change in the sugar content had been produced by doses of half and three-fourths of the amount administered in the subsequent experiment, a marked hyperglycaemia resulted. To account for this contradictory result, no very satisfactory explanation can be offered. The rabbit certainly showed considerable malaise and symptomatic disturbance very shortly after receiving the injection. Considerable difficulty was experienced in obtaining the requisite amount of blood from the auricular vein, and this may have been partly due to a concentration of the blood. This possibility would in itself tend to raise the percentage figures of the sugar content; and in this connection it may also be mentioned that owing to a similar difficulty in obtaining sufficient blood from the veins in Experiment IX it was drawn directly from the heart.

It is thus evident that after the administration of a suitable dose of hydrazine a hypoglycaemia results which is invariably associated with creatinuria, while if no change in the sugar content is produced, creatine is not found in the urine. It has also to be noted that only those rabbits which showed this combined hypoglycaemia and creatinuria exhibited any marked symptoms of metabolic disturbance, the animals being quite dull and listless and refusing all food and drink.

Of the factors which may lead to this alteration from normal metabolism, that of starvation can be eliminated on considering the results of the two fasting experiments (X and XI). Neither of the rabbits showed any reduction in the percentage of blood sugar. On the contrary, Experiment XI showed a slight increase, while in X a distinct hyperglycaemia resulted. In both cases, as already mentioned, there was a marked excretion of creatine.

Thus, although the results of the experiments here recorded point to the intimate association of carbohydrates with the metabolism of creatine, yet the exact nature of this relationship or even its degree of importance is still uncertain. It has been shown that the creatinuria following hydrazine injection is accompanied by a condition of hypoglycaemia, while it is already known that the creatinuria of diabetes and phloridzin poisoning is associated with hyperglycaemia. On the other hand, no such constant change in the sugar content of the blood is found to accompany the creatinuria induced by fasting. Furthermore, some experiments by Underhill and Rand [1910] show that "The perverted creatine metabolism as well as the metabolism of the other nitrogenous constituents of the urine tend rapidly to resume the normal on the rectal administration of dextrose, without necessarily exerting any influence on the pathological condition of the patient."

Nor do the various attempted explanations of the influence of carbohydrates on the excretion of creatine carry us very far. There is no definite evidence in favour of the hypothesis that they are essential for the conversion of creatine into creatinine or other as yet unknown substances, or for its oxidation and excretion as urea. No deductions in this connection can be drawn from previous work on the subject [MacAdam, 1913] because the creatinuria of these experiments was associated with an increased excretion of urea. So many factors enter into urea excretion, that it is impossible to eliminate them satisfactorily; and analogies drawn from *in vitro* experiments to the changes occurring *in vivo* are, in regard to the present problem, essentially untrustworthy.

That an adequate supply of carbohydrates inhibits muscle disintegration and consequently prevents the excretion of creatine is merely a special phase of the more general hypothesis suggested by Mendel and Rose [1911] that "the tissue cells may not functionate properly when the normal amount of carbohydrate food is wanting, and in this case the elimination of creatine would be analogous to the production of the acetone bodies which is also inhibited by the administration of carbohydrates." Again, Krause [1913] supports the idea that creatine may be an end product of metabolism, arguing

that creatinuria may sometimes be not so much a deviation from normal metabolism as an indication that our conceptions of normal metabolism, based as they have been on somewhat limited observations, may not be comprehensive enough. Recent work on the creatinuria of childhood [Rose, 1911; Folin and Denis, 1913] and on its irregular occurrence during the menstrual cycle of normally healthy women [MacAdam, 1914] supports this plea. The excretion of creatine may be merely one of the earliest manifestations of an upsetting of carbohydrate equilibrium, and may occur before a slight exaggeration of an otherwise normal process has become a definitely pathological phenomenon.

Conclusions.

1. After hydrazine injection in rabbits, (*a*) if no alteration in the sugar content of the blood occurs, no marked creatinuria results; (*b*) but the excretion of creatine is associated with a condition of hypoglycaemia.

2. This creatinuria is not dependent on the starvation factor *per se*, for in the two fasting experiments the excretion of creatine was associated with an increased sugar content of the blood.

3. Creatinuria appears to be produced on the least disturbance of the equilibrium of carbohydrate metabolism, of which it is one of the earliest and most sensitive manifestations.

I desire to acknowledge my deep indebtedness to Prof. Noël Paton, and Dr E. P. Cathcart for much helpful advice and criticism during the progress of this work, which was done during the tenure of a Carnegie Scholarship.

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XX. THE FORMATION OF ELLAGIC ACID FROM GALLOYL-GLYCINE BY *PENICILLIUM*.

By MAXIMILIAN NIERENSTEIN.

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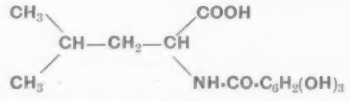
(*Received April 30th, 1915.*)

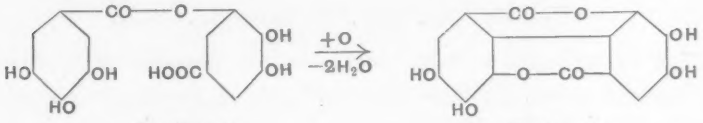
Kraemer [1900], who investigated the galls formed by *Cynips aciculata* on *Quercus cocinea*, found that during the chrysalis stage gallic acid was produced and that as the imago developed the gallic acid gave place to tannic acid. These observations gain in interest if viewed in the light of the recent investigations of v. Stockert and Zellner [1914], who have proved the presence of nitrogenous products in a number of galls caused both by insects and fungi[1], and also of the results of Nierenstein [1914] who has isolated galloyl-leucine



from the galls of *Quercus aegilops*, which are caused by *Cynips calcis*. These investigations suggest, namely, the possible formation of the digallic acid nuclei in tannic acid by way of some nitrogenous product of the galloyl-leucine type which serves as food-stuff for the growing parasites and at the same time leads to the formation of digallic acid, the presence of which in the tannic acid molecule has been established by Nierenstein [1910, 1 and 1912, 1] and which has been synthesised by Emil Fischer and K. Freudenberg [1913], so that the identification of this substance, if experimentally formed, was possible. The conditions selected as a preliminary to other investigations in progress were inquiries into the action of *Penicillium* on galloyl-glycine, which is easily prepared by the usual methods of Emil Fischer. There was, of course, the possibility, especially in the light of the beautiful investigations

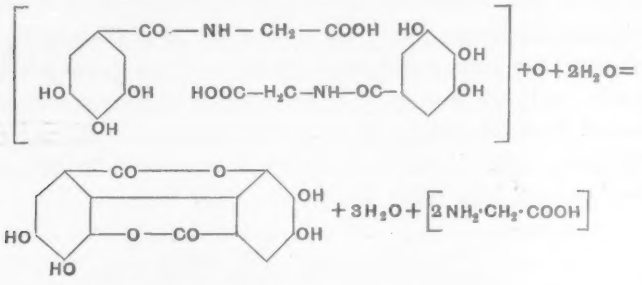
[1] With reference to gall formation see E. T. Connold [1908, 1909].

of Lewis Knudson [1913] on the action of fungi on tannic acid, that the digallic acid, if formed, would at once be decomposed into gallic acid; however, the selected conditions lead not to digallic acid, but to ellagic acid, which is a stable oxidation product of digallic acid and is frequently formed from the digallic acid nuclei of several tannins, both *in vivo* and *in vitro* [Nierenstein, 1910, 2]. It is quite possible that digallic acid is formed and subsequently converted into ellagic acid:



Digallic acid Ellagic acid

However, if one considers the above-mentioned investigations of Knudson and the pronounced oxidative properties of *Penicillium* [Herzog and Meir, 1908, 1909; Meir, 1909] it is far more likely that the elimination of the glycine radical and the ellagic acid formation through oxidation and anhydration [Nierenstein, 1912, 2] take place simultaneously:



This interpretation assumes that the glycine radical is eliminated by hydrolysis, which is hypothetical, as the present investigations have only established the formation of ellagic acid and nothing is at present known as to the fate of the glycine part.

In this connection reference is made to C. L. Berthollet [1809] and also to Merklin and Woehler [1845], who have found ellagic acid in the benzoarstones of Persian goats, which suggests that galloyl-glycine (trihydroxyhippuric acid) is, perhaps, also formed in the animal organism, especially as ellagic acid has also been found by Woehler [1848] in the bladder of *Castoreum canadense*.

It must, however, be remembered that gallic acid on administration is recovered either as free gallic acid [Stockmann, 1886, 1898; Baumann and Herter, 1877; Harnack, 1898] or in combination with sulphuric acid [Rost, 1898], but never in combination with glycine [Heffter, 1905].

Experimental.

Tricarbomethoxygalloyl-glycine.

$(CH_3—CO_2—O)_3 . C_6H_2 . CO . NH . CH_2 . COOH.$

Five grams glycine in 75 cc. water and 3·5 g. potassium hydroxide are mechanically shaken for three hours with 26·8 g. tricarbomethoxygalloyl chloride [Emil Fischer, 1908] dissolved in ether, which is added in three portions and cooled before adding. The solution is then left for two to three hours in a cooling mixture and acidified with carefully cooled hydrochloric acid after separation from the ether. The product thus obtained crystallises from alcohol in prismatic needles, which melt at 202°–204° with decomposition and evolution of carbon dioxide. The substance is also soluble in acetone and ethyl acetate. The yield is 94 %.

In addition to the usual analysis an hydroxyl estimation by the method of Daniel and Nierenstein [1911] was also made. As in other cases, where the hydroxyls were in the meta-position to the carboxyl groups [Daniel and Nierenstein, 1911; Nierenstein, 1912, 3], nearly 2 % more carbon dioxide was formed than required by theory. This increase is derived from the carboxyl group, which is partly eliminated in these cases by the pyridine [see Bredig and Fajans, 1910; Nierenstein, 1912, 4].

Analysis. 0·2577 g. : 8·3 cc. N_2 at 19° and 767 mm.
0·1850 g. : 0·2816 g. CO_2; 0·0565 g. H_2O[1]
0·2462 g. : 0·0708 g. CO_2; (Daniel and Nierenstein)

		Calculated for $C_{15}H_{15}O_{12}N$
N	3·74 %	3·49 %
C	42·49 %	42·76 %
H	3·42 %	3·69 %
CO_2	28·86 %	26·92 %

Galloyl-glycine

$(OH)_3C_6H_2 . CO . NH . CH_2 . COOH.$

Five g. tricarbomethoxygalloyl-glycine suspended in water are treated on the water-bath at about 40° with 50 cc. of a 10 % solution of pyridine

[1] The combustion was carried out in oxygen [see Francis and Nierenstein, 1911].

and water. The evolution of carbon dioxide is finished in about two hours, when the solid is crystallised from absolute alcohol. It is obtained in cubes, which melt at 282°–283°, the yield being 97–98 %. Galloyl-glycine gives like galloyl-leucine [Nierenstein, 1914] only the iron chloride reaction, but not the potassium cyanide reaction for gallic acid [Sidney Young, 1883].

Analysis. 0·2399 g. : 13·3 cc. N_2 at 18° and 755 mm.
0·1758 g. : 0·3057 g. CO_2; 0·0572 g. H_2O

		Calculated for $C_9H_9O_6N$
N	6·36 %	6·23 %
C	47·32 %	47·59 %
H	3·64 %	3·89 %

Three g. galloyl-glycine were hydrolysed with dilute hydrochloric acid (10 %) in a sealed tube and the solution extracted with ether. The ethereal part gave on evaporation gallic acid, which had the correct melting point and gave all the specific reactions for gallic acid. The aqueous solution was benzoylated; the hippuric acid obtained melted at 185°–186° and caused no depression when a mixed melting point with hippuric acid was carried out.

Formation of ellagic acid from galloyl-glycine.

Five g. galloyl-glycine and 2 g. sodium bicarbonate are dissolved in 250 cc. of water and the solution saturated with carbon dioxide. The sterilised solution is then inoculated with *Penicillium* (*spec.* ?) grown in a solution of tannic acid, which contains free sugar [see Geake and Nierenstein, 1914]. The solution becomes cloudy after standing in an incubator for 32 hours at 42° and in about 78 hours a precipitate begins to be formed. After an incubation of 22 days, the *Penicillium* is carefully removed and the precipitate collected and washed with dilute hydrochloric acid. The product gives the specific Griessmayer reaction for ellagic acid [Nierenstein, 1909] and crystallises from pyridine in prismatic needles, which contain pyridine [see Perkin and Nierenstein, 1905]. The yields of three preparations were 83 %, 87 % and 84·5 %.

For analysis the product was treated with alcohol and dried at 160°.

Analysis 0·1946 g. : 0·3958 g. CO_2; 0·0395 g. H_2O

		Calculated for $C_{14}H_6O_8$
C	55·50 %	55·62 %
H	2·27 %	1·98 %

The acetylellagic acid prepared according to Perkin and Nierenstein [1905] crystallised from alcohol in glistening, colourless, flat needles, which

commenced to sinter at about 331° and melted at 341°–344°, which agrees with the melting point given by Perkin and Nierenstein.

Analysis. 0·1780 g. : 0·3679 g. CO_2; 0·0489 H_2O

	Calculated for $C_{14}H_2O_8(CO{-}CH_3)_4$
C 56·37 %	56·17 %
H 3·06 %	2·98 %

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XXI. THE ACTION OF NORMAL AND BASIC LEAD ACETATE ON THE SUGARS, WITH REMARKS ON RUBNER'S TEST FOR DEXTROSE AND LACTOSE.

By HAROLD ROGERSON.

From the Chemical Department, Guy's Hospital Medical School.

(*Received May 6th, 1915.*)

During the course of some experiments on diabetic urines, Dr E. L. Kennaway observed the formation of a pink colouration in many cases when the urines had been treated with basic lead acetate and ammonia for the removal of dextrose. The colouration developed very gradually, and after two days' interval, a pink precipitate separated, leaving the solution almost colourless. As noted, the colouration was not obtained in every instance, and it was inferred either that the colouration was due to the so-called "Rubner reaction" indicating the incomplete removal of dextrose, or that some pathological substance might be present.

The author, at the suggestion of his colleague, conducted some experiments in order to elucidate this point and found that the colouration was due to dextrose.

It was deemed advisable to ascertain how other sugars behaved towards normal and basic lead acetate under different conditions, not only with and without ammonia but also other alkalies. The results obtained indicate that "Rubner's test" for dextrose and lactose is given equally well by many other sugars.

The normal lead acetate used was a saturated solution, and the basic solution was prepared according to the British Pharmacopoeia.

I (*a*). *Normal lead acetate alone at ordinary temperature.*

1 cc. sugar solution (10 %).
10 cc. saturated lead acetate.

Dextrose	No change in 12 days.
Laevulose	Yellow in 5 days, darker in 12 days.
Galactose	Faint yellow in 1 day, gradually darker to 12th day.
Mannose	No change in 12 days.
Lactose	,, ,,
Sucrose	,, ,,
Maltose	,, ,,
Arabinose	Similar to galactose

The dextrose and galactose used in the above experiment had been soaked in alcohol for eight years, so there can be no doubt about the purity of these two sugars. If the above Table I (*a*) be compared with the Table I (*b*) it will be seen that the results depend almost entirely on the temperature. No precipitates were formed in any of the above cases.

I (*b*). *Solutions heated just to boiling.*

Dextrose	Yellow at 60°.	Orange-yellow at 100°.	No ppt.
Laevulose	,, ,,	Lighter orange-yellow.	,,
Galactose	,, ,,		Ppt.
Mannose	,, ,,		,,
Lactose	,, ,,		Very slight ppt.
Sucrose	No colour.		No ppt.
Maltose	Light yellow at 60°.	Orange-yellow at 100°.	Very slight ppt.
Arabinose	,, ,, 80°.	Darker yellow at 100°.	No ppt.

The above results indicate that the reaction alone with normal lead acetate would make it somewhat difficult to ascertain the presence of any individual sugar. Sucrose is of interest because it does not show any colour either at the ordinary temperature or on boiling the solution. This is the case also when basic lead acetate is used; indeed, sucrose is the only sugar which did not give any results which could be noted.

II (*a*). *Basic lead acetate alone at ordinary temperature.*

Dextrose	Very faint yellow in ½ hr.,	orange	in 3 days,	yellow after	18 days.
Laevulose	Light yellow ,,	yellow	,,	yellow	,,
Galactose	Orange-yellow ,,	orange	,,	orange	,,
Mannose	No change ,,	no change (slight ppt.)	,,	faint yellow	,,
Lactose	No change ,,	faint yellow	,,	yellow after	,,
Sucrose	No change ,,	no change	,,	no change	,,
Maltose	Opalescent ,,	yellowish-pink	,,	yellow	,,
Arabinose	Cf. galactose ,,	yellow	,,	yellow	,,

II (*b*). *Solutions heated just to boiling.*

Dextrose	Faint yellow at 50°. Dark yellow at 100°. No ppt.
Laevulose	„ „ „ „ Slight ppt. on cooling.
Galactose	Orange-yellow at 40°. Very dark at 70°. Lighter at 100°. Ppt.
Mannose	No colour before 100°. Yellow rapidly, then darker. Initial ppt.
Lactose	Same as mannose but no initial ppt.
Sucrose	No colour. No ppt.
Maltose	Faint yellow at 75°. Lemon-yellow at 100°, then darker.
Arabinose	Yellow at 30°. Then same as galactose.

If Tables I (*b*) and II (*b*) be compared it will be seen that the colourations obtained in the latter case are much more pronounced than those obtained in the former. The same results were observed also when the sugars were treated with the above reagents in the presence of alkalies.

III (*a*). *Normal lead acetate with ammonia.*

At the ordinary temperature, this was done with dextrose only, when the following result was obtained:

10 cc. dextrose (10 %) and 200 cc. lead acetate (7·5 %); added NH_4OH (0·880) until a slight precipitate formed which occurred after 38 cc. had been used.

After 75 minutes, a faint pink colouration was evident, which became darker until, after five days, the solution became cloudy and a copious pink precipitate separated, leaving the liquid almost colourless.

III (*b*). *Solutions heated just to boiling.*

1 cc. sugar (10 %).
1 cc. NH_4OH (0·880).
10 cc. saturated lead acetate.

Dextrose	Pink at 45°, orange-red at 100°. No immediate precipitate.
Laevulose	Yellow at 45°, darker and then lighter at 100°; again darker.
Galactose	„ „ then same as dextrose.
Mannose	No colour before boiling, then pink to dark yellow.
Lactose	Faint yellow at 55°, darker at 100°, then reddish-brown.
Maltose	„ „ 75°, orange „ then opalescent.

Rubner's test for Dextrose and Lactose.

This test [Rubner, 1885] is carried out by adding some lead acetate (the ordinary laboratory reagent) to a dilute solution of dextrose, and then adding ammonia, drop by drop, until a permanent precipitate is formed.

On standing in the cold the liquid becomes coloured gradually, first yellow and then rose or flesh red; on heating the colouration takes place more quickly. It is also stated that the addition of the reagents must take place in the order named.

A series of tests was accordingly performed according to the directions given by Rubner, when the following effects were noted:

IV.

10 cc. sugar (1 %).
1 cc. normal lead acetate (about 10 %).
NH_4OH until permanent precipitate.

On warming,	Dextrose	gave flesh colour and precipitate.
	Laevulose	„ „ „
	Galactose	„ „ „
	Mannose	„ yellow colour but no precipitate.
	Lactose	„ „ „ and precipitate.
	Maltose	„ „ to buff colour and precipitate.

The observations, as noted in Table IV, clearly indicate the futility of adopting this reaction as a specific test for dextrose and lactose, and consequently it should be deleted from the literature.

It was deemed of interest to ascertain the effects of organic liquids on the coloured solutions and precipitates obtained in the above-described reactions. On shaking the solutions, in which a colouration had developed, with ether or chloroform the colour disappeared; but, on keeping, the colouration was gradually restored, and on renewed shaking did not entirely disappear.

The effect of using alcohol was then tried. A saturated 90 per cent. alcoholic solution of dextrose was treated with alcoholic solutions of lead acetate and ammonia, when a colouration ranging from pink to orange-red was obtained on standing at the ordinary temperature. The precipitate first formed dissolved in the excess of lead acetate solution added, but no precipitate was subsequently thrown down, and the colour was confined solely to the solution.

The behaviour of basic lead acetates towards the sugars is much more sensitive than that of the normal salt, and the experiments to be described have been extended to most of the sugars one would be likely to meet with in biochemical investigations. Other alkalies besides ammonia were tried, as well as acetates of other metals, but in the latter case no definite effects were observed.

V (*a*). *Dextrose, basic lead acetate and ammonia at the ordinary temperature.*

10 cc. dextrose (10 %).
5 cc. NH_4OH (0·880).
100 cc. basic lead acetate (B.P. fortis).

After 2 minutes	Yellow tint	Solution clear
1 hour	Salmon pink	,, ,,
2 hours	Blood-red	,, ,,
3·5 ,,	Darker red	,, ,,
21·5 hours	Very dark red	,, ,,
3 days	Lighter red	Pink precipitate
14 ,,	Dark yellow	Pink and buff precipitate

It will be seen from the above Table V (*a*) that the colour goes to a maximum before any precipitate forms; and it was also observed by using 10, 5, and 1 per cent. solutions that the amount of precipitate actually formed on mixing the solutions was proportional to the amount of sugar present, as was also the maximum tint developed. Therefore the colour is proportional to the sugar-strength of the solution. Other sugars and polyhydric alcohols gave the following results:

V (*b*).

Laevulose (m.p. 100)	Same as dextrose	Pink precipitate
Galactose (m.p. 144)	Same as dextrose	Pink ,,
Mannose	No colour	White cryst. precipitate
Lactose	Reddish-orange	Pink precipitate
Maltose	Yellow	Buff ,,
Sucrose	No colour	No ,,
Arabinose	Dark orange	Buff ,,
Xylose	Slight pink	Pink ,,
α-Methyl glucoside	No colour	White cryst. precipitate
Dulcitol	,,	White amorph. ,,
Mannitol	,,	No precipitate

The results shown in the above Table V (*b*) indicate that it would be almost impossible to identify any particular sugar tested under the above conditions at ordinary temperature. A full series of observations were then made with various sugars, the solutions being raised to boiling, and the temperature noted at which any colouration first appeared.

The following table shows the results obtained for dextrose.

V (*c*).

Experiment	Dextrose (10 %) in cc.	H_2O in cc.	$NH_4 \cdot OH$ (·880) in cc.	Basic lead acetate in cc.	Solutions before heating	Effect on heating
1.	1·0	0·0	1·0	10·0	Clear after few seconds	Pink at 45°. Then darker.
2.	0·5	0·5	1·0	10·0	Clear at once	„ 55° „ „
3.	0·1	0·9	1·0	10·0	„ „	Pink at 65°. Then yellow and white cryst. ppt. slowly forms.
4.	0·1	0·9	0·5	5·0	„ „	Pink at 63°. Darker yellow than (3).
5.	0·1 (1 %)	0·9	0·1	0·9	„ „	Pink at 62°. Darker than (3) or (4), but does not turn yellow.
6.	1·0	0·0	1·0	10·0	„ „	Pink at 65°. Then yellow (cf. 3).
7.	0·5	0·5	1·0	10·0	„ „	Pink at 75°. Yellow at 80°, white cryst. ppt. on standing.
8.	0·1	0·9	1·0	10·0	„ „	No colour. White cryst. ppt. on boiling. Almost solid.
9.	0·0	0·0	1·0	10·0	„ „	Same as 8.
10.	0·1	0·9	0·5	5·0	„ „	No colour. White cryst. ppt. on boiling, but not large.
11.	0·1	0·9	0·1	0·9	„ „	Yellow at 95°. Slight ppt.

The above results in Table V (*c*) clearly indicate the sensitiveness of the reaction. The white crystalline precipitate obtained in 8, 9, and 10 is of interest since this is known to be a basic lead acetate of definite composition, and the fact that it is only formed when the sugar is present only in very small amount shows that dextrose (and other sugars) exerts an inhibitive effect on its formation.

A corresponding series of experiments was performed with all the other sugars and the results obtained were much of the same order. Only one test with each is quoted in the following table, the quantities used being the same as in the first experiment in Table V (*c*).

VI.

Sugar	Solution before heating	Effect on heating
Laevulose	Solution not quite clear	Yellow, then pink, clear at 70°, then dark orange on boiling.
Galactose	Solution clears slowly	Yellow at 30°, orange-pink at 65°, pink ppt. separates.
Mannose	„ „ „	No colour before boiling, then yellow. No pink, orange or ppt.
Lactose	Considerable ppt.	Faint yellow at 60°, yellow on boiling. Rapid pink ppt. Solution orange.
Maltose	„ „	Opalescent on warming, yellow on boiling, then pink, and orange. Pink ppt.
Sucrose	Solution clears slowly	No colour and no ppt.

In describing his test, as previously noted, Rubner directs the reagents to be added in a definite order, viz., to the sugar solution add lead acetate and then ammonia. The above experiments, however, show that it is quite immaterial whether the lead solution or the ammonia is added first, the difference being that, if the ammonia is added first, the initial precipitate formed on adding the lead solution is much greater than if the solutions are mixed in the alternative order. In any case the result is the same.

Basic lead acetate and different alkalies with dextrose.

It was deemed of interest to see how other alkalies would behave, and the hydroxides of sodium and barium were accordingly tried with dextrose in the presence of basic lead acetate. The results, which were obtained at the ordinary temperature, may be thus summarised:

VII.

NaOH	After 15 minutes, white precipitate.
	After 50 minutes, slight pink colouration evident.
	After 15 hours, orange. No more precipitate.
$Ba(OH)_2$	Fugitive yellow which became permanent after 7 hours.

The action of different acetates with dextrose and alkali.

Since lead acetate gives intense colourations with many sugars, it was decided to see if the acetates of other metals gave similar reactions. The acetates of the following metals were used: barium, zinc, mercury, manganese, cadmium and uranium. Of these, the only acetate which showed any colouration was that of barium, when the solution was at the ordinary temperature. In this case the following results were obtained:

VIII.

$Ba(C_2H_3O_2)_2$ and $Ba(OH)_2$	After 1 day, yellow colour, no precipitate.
„ „ NH_4OH	„ 4 days, faint yellow, no precipitate.

The action of alkalies alone on the sugars.

One of the characteristic tests for dextrose is the action of sodium or potassium hydroxide (Moore's reaction), and barium hydroxide behaves similarly. Therefore in performing the tests described above where NaOH and $Ba(OH)_2$ were used, the effects observed at the ordinary temperature have been noted. Moreover, the present author has observed that if solutions of various sugars are boiled with ammonia for a short time, colourations

varying from pink to yellow are formed. The following results were obtained with 10 per cent. solutions, and boiling for a few minutes with about an equal volume of ammonia (0·880).

IX.

Dextrose	Faint yellow in 1 minute. Distinct in 3 minutes.
Laevulose	" " " " "
Galactose	" " " " "
Mannose	Very faint yellow after 3 minutes.
Lactose	Cf. dextrose. Yellowish-pink on standing.
Maltose	Faint yellow in 1 minute. Orange in 3 minutes.
Arabinose	No change.

It will be apparent from the above observations that the combined action of the acetates of lead and ammonia is somewhat complex, and it is impossible to correlate the effects observed with any particular sugar in order to identify it. No quantitative experiments have been carried out up to the present, but there is little doubt that the precipitates obtained in many cases are complex derivatives of the sugar and the acetates of lead.

REFERENCE.

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XXII. THE QUANTITATIVE DETERMINATION OF GLYOXALASE IN THE BLOOD AND SOME OF ITS APPLICATIONS.

By HAROLD WARD DUDLEY.

From the Animal Nutrition Research Institute, University of Leeds.

(*Received May 8th, 1915.*)

In previous communications [Dakin and Dudley, 1913, 1914] the properties of the enzyme glyoxalase, which converts α-ketonic aldehydes into α-hydroxy-acids according to the general scheme:

$$R . CO . CHO + H_2O = R . CHOH . COOH$$

have been discussed. It has been demonstrated that the enzyme, which is widely distributed, rapidly converts methylglyoxal into lactic acid, and is therefore most probably of importance in the intermediary metabolism of carbohydrates. In order to investigate more closely some problems connected with the enzyme a quantitative method of estimation was desirable, and the object of the present paper is to describe such a method and some of the results obtained by its aid.

On account of the difficulty of the quantitative extraction of lactic acid from liquids containing protein and the laboriousness of its determination, methylglyoxal could not be used with any hope of success as substrate in a quantitative method for estimating glyoxalase activity. The previous qualitative work on the enzyme was greatly facilitated by the employment of phenylglyoxal as substrate. The ease with which the optically active mandelic acid produced by the action of the enzyme can be extracted from digestion mixtures containing protein and the high value of the specific rotation of the acid, render quick determinations of the activity of glyoxalase preparations possible. Accordingly a quantitative method was devised, using phenylglyoxal as substrate, which was in principle essentially the same as the method employed in previous qualitative work on the enzyme.

The primary object of the quantitative estimation of glyoxalase was the comparison of its activity in the living animal under varying conditions in the hope of gaining more detailed knowledge as to its function in carbohydrate metabolism. Blood was therefore chosen as the tissue in which to determine the activity of the enzyme, since its withdrawal from time to time in small quantities is without effect on the general condition of the animal, and comparisons between equal volumes of different specimens can be accurately made.

The method consists in allowing 5 cc. of defibrinated blood to act on phenylglyoxal in the presence of calcium carbonate at 37° for 20–24 hours. The protein is then precipitated by adding solid ammonium sulphate and heating. The mixture, after acidification, is filtered, and the protein precipitate is washed free from mandelic acid, which is then extracted from the filtrate with ether and determined polarimetrically.

It was found at the outset that the concentration of phenyglyoxal is by no means a matter of indifference. It exhibits a toxic effect on the enzyme in concentrations even as low as 0·80 %. It is therefore necessary to have a standard concentration in all quantitative determinations. It was further shown that the method is not applicable without modification to amounts of blood greater than 7 cc., mainly because of the difficulty of washing the bulky protein precipitate free from mandelic acid. Amounts of blood from 1 to 7 cc. give amounts of active mandelic acid, measured polarimetrically, in simple proportion to the quantity of blood taken.

By means of this method the fact has been established that the "glyoxalase value" for the blood of different animals of the same species is remarkably constant, and is also definite for the particular species. Comparisons between species show definite differences in the glyoxalase values of the blood. In descending order of glyoxalase activity the species so far examined arrange themselves thus:

Dog, Ox, Rabbit, Sheep, Pig.

The order in which these animals appear is interesting. There appears to be a rough parallelism between the glyoxalase activity and the sugar tolerance of the animals. It has been shown by Hunter and Hill [1914] that "the sheep's capacity to deal with subcutaneously administered glucose is greatly inferior to that of the dog," while Carlson and Drennan [1912] have observed an extraordinarily low sugar tolerance in the case of the pig. It will be noticed that the sheep and the pig show the lowest values for blood

glyoxalase in the various species so far examined, whilst the value for the dog is the highest. Experiments of Underhill and Closson [1906] have demonstrated that the dog can utilise large quantities of glucose introduced parenterally without developing a glycosuria. They have shown, too, that the rabbit is able to assimilate much glucose injected subcutaneously; indeed under the experimental conditions observed by them its sugar tolerance appears to be similar to that of the dog. But apparently no accurate determinations of the limits of sugar tolerance in these animals have been made. If the glyoxalase of the blood is any guide to the sugar tolerance of an animal one would expect the rabbit to occupy an intermediate position between the dog and the sheep. It certainly can assimilate more parenterally administered glucose than the sheep, but its efficiency as compared with the dog has not been determined.

Without doubt the sugar tolerance of an animal must be governed by the interplay of many complicated factors, and of these glyoxalase activity may be one, so that the apparent parallelism between the sugar tolerance of an animal and the glyoxalase value of its blood may not be entirely fortuitous.

Experiments on the glyoxalase activity of the blood in various forms of glycosuria are in progress and will be reported shortly.

Experimental.

Quantitative determination of glyoxalase in 5 cc. of blood.

Before the blood is shed a 250 cc. conical flask containing 10 cc. of a suspension of 2·75 g. chalk in 100 cc. water is placed in an incubator at 37°. A 1·2 % solution of phenylglyoxal hydrate is also heated to 37°. The experimental animal is then bled and the blood is whipped until free from fibrin, when it is strained through muslin. 10 cc. of the phenylglyoxal solution (0·12 g. phenylglyoxal hydrate) are then well mixed with the chalk suspension and 5 cc. of the blood are finally added. It is important to make the additions in this order since most phenylglyoxal preparations give distinctly acid solutions. This acidity is removed by the chalk before the blood is added and so can do no damage to the glyoxalase, which has been shown to be very sensitive to acid. The mixture is then incubated at 37° for 20–24 hours. Experiments have been performed which show that the action is complete in this period. Phenylglyoxal displays distinctly antiseptic properties, so that the addition of toluene is unnecessary. In no case in the performance of many experiments has any bacterial activity been

observed. After completion of the reaction 25 g. ammonium sulphate are added to the mixture, which is then heated in a boiling water-bath for 3–4 minutes. The protein is thereby precipitated. After cooling, 12 cc. of 50 % phosphoric acid are added and the liquid is allowed to stand, with frequent shaking, for half an hour. The protein precipitate is then filtered on a Buchner funnel and washed five times with 10 cc. 40 % ammonium sulphate solution. These washings must be performed slowly, each washing being allowed to soak well into the precipitate before being sucked through. A water-clear filtrate is obtained which is extracted four times with small quantities of ether (10–12 cc.). Each ether layer is washed with distilled water (about 3 cc.). The mandelic acid is completely removed by this process. The ether is then removed by distillation and a crystalline residue of mandelic acid is obtained. This is dried in a desiccator over sulphuric acid and then dissolved in 12 cc. distilled water, solution being accelerated by warming on a water-bath. A pinch of animal charcoal is added to the hot solution, which is first allowed to cool and is then filtered. The small amount of fat which usually appears in the ether residue is completely removed by these means. The solution is then examined polarimetrically in a 2 dm. tube, the reading giving a measure of the amount of *l*-mandelic acid formed, and so of the glyoxalase content of the blood.

Experiments to show the effects of increasing concentration of phenylglyoxal and of dilution, and the completeness of removal of mandelic acid from the protein precipitate.

Sheep's blood was used in this series. The technique was that described in the standard method outlined above.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	Total vol. cc.	α	*l*-mandelic acid, g.
1.	10	30	5 (0·1 g.)	5	50	−0·66°	0·0251
2.	10	25	10 (0·2 g.)	5	50	−0·62°	0·0235
3.	10	25	10 (0·2 g.)	5	50	−0·63°	0·0239
4.	10	15	20 (0·4 g.)	5	50	−0·45°	0·0171
5.	10	75	10 (0·2 g.)	5	100	−0·60°	0·0228

After the usual filtration and washing of the precipitate in Experiment 2, the protein was subjected to five further washings by 10 cc. 40 % ammonium sulphate solution and these washings were extracted four times with ether. No mandelic acid was obtained by this treatment, showing that the acid had been completely removed by the previous manipulations.

Experiments 2 and 3 serve to demonstrate how closely duplicate determinations agree.

Experiment 4 shows definitely the toxic effect of higher concentrations of phenylglyoxal on the enzyme.

Experiment 5 shows that dilution of the reaction mixture to twice the volume taken in standard experiments is without appreciable effect on the result.

Experiments showing the applicability of the method for amounts of blood from 1 to 7 cc. and its failure for larger quantities.

The first series was carried out with ox blood, the second with sheep's blood. I am indebted to Dr H. E. Woodman for the second series.

OX BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	α	*l*-mandelic acid, g.
1.	10	29	10 (0·2 g.)	1	−0·19°	0·0072
2.	10	26	10 (0·2 g.)	4	−0·88°	0·0334
3.	10	25	10 (0·2 g.)	5	−1·04°	0·0395
4.	10	25	10 (0·2 g.)	5	−1·03°	0·0391
5.	10	23	10 (0·2 g.)	7	−1·31°	0·0497
6.	10	20	10 (0·2 g.)	10	−1·06°	0·0403
7.	10	15	10 (0·2 g.)	15	−0·84°	0·0319
8.	10	10	10 (0·2 g.)	20	−0·56°	0·0213
9.	Protein precipitate of 8, further washed with 50 cc. 40 % ammonium sulphate solution, filtrate extracted with ether: a quantity of mandelic acid was extracted which, examined under standard conditions, gave $\alpha = -0·40°$.					

SHEEP'S BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	α	*l*-mandelic acid, g
1.	10	29	10 (0·1 g.)	1	−0·12°	0·0045
2.	10	27	10 (0·1 g.)	3	−0·35°	0·0133
3.	10	25	10 (0·1 g.)	5	−0·63°	0·0239
4.	10	23	10 (0·1 g.)	7	−0·79°	0·0300
5.	10	21	10 (0·1 g.)	9	−0·57°	0·0216

Experiments 1 to 5 of the first series and 1 to 4 of the second show that the amount of *l*-mandelic acid produced is proportional to the amount of enzyme taken. The effect of adsorption of mandelic acid by the protein precipitate is beginning to be felt when 7 cc. of blood are taken, and with higher quantities the results are of no value.

Experiments showing the rate of the reaction under the conditions of the standard determination.

A series of experiments, using 5 cc. of dog's blood in each, carried out according to the standard method, were interrupted at various intervals and afforded information with regard to the progress of the reaction.

Action stopped after	1	2	4	6	10	20	23 hours
$\alpha =$	−0·28	−0·39	−0·57	−0·67	−0·83	−0·99	−0·99°

It will be seen that the velocity of the reaction is large at first, diminishing considerably in the later stages, and that under the conditions of experiment the reaction is complete in 20 hours.

Normal glyoxalase values for various animals.

5 cc. of freshly-shed, defibrinated blood were used in all these determinations, which were carried out in accordance with the standard method.

Dog. The following rotations for *l*-mandelic acid obtained by the action of 5 cc. dog's blood in the cases of seven different animals, most of the experiments being performed in duplicate, serve to show how constant is the value of the glyoxalase content of the blood in a particular species. The dogs, some male and some female, were of different breeds.

Dog	1	2	3	4	5	6	7
$\alpha =$	−1·05° −1·04°	−1·04°	−1·02° −1·03°	−1·08° −1·00°	−1·07° −1·06°	−1·03° −1·03°	−1·05°

Mean value for $\alpha = -1{\cdot}04° = 0{\cdot}0395$ g. *l*-mandelic acid.

Ox. The blood of two animals was investigated.

Mean value for $\alpha = -1{\cdot}03° = 0{\cdot}0382$ g. *l*-mandelic acid.

Rabbit. Four animals were used.

Mean value for $\alpha = -0{\cdot}74° = 0{\cdot}0281$ g. *l*-mandelic acid.

Sheep. Duplicate determinations were made on the blood of two animals.

Mean value for $\alpha = -0{\cdot}63° = 0{\cdot}0239$ g. *l*-mandelic acid.

Pig. Two animals were investigated.

Mean value for $\alpha = -0{\cdot}61° = 0{\cdot}0232$ g. *l*-mandelic acid.

SUMMARY.

A method for the quantitative determination of the glyoxalase activity of blood is described.

It is shown that the glyoxalase of the blood is remarkably constant in different animals of the same species under normal conditions. It is further demonstrated that different species display definite differences in the glyoxalase activity of the blood, and attention is drawn to the observation that animals displaying high glyoxalase values are those having high sugar tolerance, whilst those with lower glyoxalase activities have less well developed powers of assimilating sugar.

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XXIII. THE FORMATION OF HUMIC BODIES FROM ORGANIC SUBSTANCES.

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The true nature of the dark brown and black substances formed by the decomposition of organic matter in the soil, and grouped together under the collective name of humus, has long been a problem to both chemists and biologists, owing to the fact that the composition of these substances varies not only with the nature of the humus-producing materials, but also with the temperature, moisture and pressure relations, and the methods of extraction.

A study of the extensive literature which deals with humus shows that the widely divergent opinions expressed and the inconsistent results obtained group themselves around two chief views regarding the nature of humus.

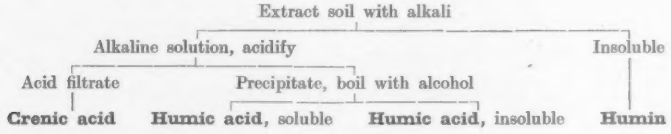
The first view is that humus has a simple composition and consists of one or a few definite chemical substances. De Saussure [1804] describes it as a "brown combustible powder soluble in alkalies and ammonia compounds." Mulder [1849] says that it consists of seven different organic substances—crenic acid, apocrenic acid, geic acid, humic acid, humin, ulmic acid and ulmin. He considered these substances to be intimately related, and five at least were distinct steps in the decay of organic matter in the soil.

This view of the simple composition of humus was the dominant one amongst the earlier writers on the subject; and we find it existing even to-day in a slightly modified form in most agricultural text-books. In these books humus is regarded either as a more or less definite substance which can be obtained by extracting the soil with a dilute alkali after first treating with a dilute acid to remove the lime which renders the humus insoluble, or as being synonymous with the so-called humic acid which can be precipitated from alkaline solutions of the soil.

The second view, that humus is of a very complex nature, was first advanced by Hermann [1842]. He laid special stress on "the presence of a great number of various kinds of humus-substances," and divided these various substances into three groups: *humus extract*, soluble in water; *humic acid*, soluble in alkalies and precipitated by acids; *humin*, insoluble in water and alkalies.

Hoppe-Seyler [1889] added another group which he calls the *phlobaphene* group, comprising those substances dissolved out by alcohols from the acid precipitates of the humic acid group.

Still more recently Schreiner and Shorey [1910] have given a somewhat similar grouping for the various compounds they have isolated from the organic matter of soils. They, however, include another group—the *crenic acid* group—comprising the substances isolated from the acid filtrate obtained when an alkaline solution of the soil is treated with mineral acid. Their grouping may be illustrated as follows:



Schreiner and Shorey have isolated and identified 20 distinct compounds from the organic matter of soils, only one of which—a pentosan—is a carbohydrate. These substances have all been obtained from the crenic acid and alcohol-soluble humic acid groups, and nothing is known as to the identity of the constituents of the humin and alcohol-insoluble humic acid groups. Yet they state that these last two groups, the components of which are unknown, contain 39·8 per cent. of the total organic carbon found in soils.

When one considers that carbohydrates, represented by sugars, starch and celluloses, make up by far the greater portion of the organic substance of plants and so form the greater part of the material from which soil humus is derived, it appears strange that so far only one carbohydrate has been isolated from soil, and the suggestion naturally arises that there may be some connection between carbohydrates and these unknown components of the humic acid and humin groups.

The probability of such a connection was indicated during a research as to the value of certain kinds of so-called "humates" as a nutrient for the nitrogen-fixing organism *Azotobacter*, and the present paper describes the results of an investigation of the relationship between these substances.

It has been pointed out that the terms humic acid and humin are used with varying connotation by different writers. For the purpose of this investigation it has been assumed that the terms refer to two groups of humic substances: *humic acid*—substances thrown down as brown colloidal precipitates by mineral acids from the water or alkaline extracts of humus; *humin*—substances insoluble in water and alkalies, but rendered soluble by fusing with caustic soda or potash, from the solution of which humic acid can again be precipitated.

Humic Bodies from Sugars by the action of Mineral Acids.

The discovery by Malaguti [1858] that humic acid and humin are formed when sugar is boiled with either hydrochloric or sulphuric acid was important as indicating a possible origin for these substances in the soil, but no attempt was made to trace their inter-relationship.

Later, Conrad and Guthzeit [1885] stated that on boiling raw sugar with dilute hydrochloric acid it is first changed into dextrose and laevulose, and that then the dextrose forms humic acid and the laevulose humin. As these authors give no experimental proof of the accuracy of their statement, an experiment was made to test the effect of boiling sucrose, dextrose and laevulose separately with hydrochloric acid. Five grams of each were boiled with a 7·5 per cent. solution of hydrochloric acid under a reflux condenser for 30 minutes. All three yielded a brown deposit which on testing proved, in each case, to be a mixture of humic acid and humin. The relative proportions, however, of these two substances in the three mixtures showed considerable variation, and the proportion of humic acid to humin always diminished as the boiling continued. It was found that with a strong solution of hydrochloric acid the conversion of humic acid into humin took place rapidly. In order therefore to trace in detail the changes taking place, a weak solution of the acid was employed. Five g. of sucrose, dextrose and laevulose respectively were boiled with a 3 per cent. solution of hydrochloric acid under a reflux condenser for varying periods of time. As is well known, the colourless solutions on heating pass through a series of colour changes: yellow, orange, red, brown, black, and with the brown colour the formation of a deposit is first noticed. Laevulose and sucrose pass through these changes fairly rapidly, but with dextrose the rate of change is much slower.

Laevulose:	Yellow	1	minute;	red	5	minutes:	brown	8 minutes.
Sucrose:	,,	2	minutes	,,	8	,,	,,	12 ,,
Dextrose:	,,	11	,,	,,	60	,,	,,	90 ,,

The first deposit obtained in each case was found to be humic acid without a trace of humin. On further boiling the humic acid becomes changed into humin. The proportions obtained by boiling for periods of 0·25, 0·5, 1, 2 and 3 hours are given in the following table:

	15 mins.	30 mins	1 hr.	2 hrs.	3 hrs.
5 g. sucrose; total ppt.	0·106	0·077	0·140	0·254	0·405
„ humic acid	0·007	0·040	0·101	0·148	0·157
„ humin (by diff.)	0·009	0·037	0·039	0·106	0·248
5 g. dextrose; total ppt.	No deposit	No deposit	No deposit	0·003	0·011
„ humic acid				0·002	0·005
„ humin (by diff.)				0·001	0·006
5 g. laevulose; total ppt.	0·028	0·156	0·244	0·492	0·685
„ humic acid	0·010	0·103	0·104	0·184	0·165
„ humin (by diff.)	0·018	0·053	0·140	0·308	0·520

From the above results it is seen that as the boiling is prolonged the proportion of humic acid to humin decreases, although the total amount of the deposit increases. This suggests that humic acid is an intermediate stage in the conversion of sugars into humin. That humic acid is directly convertible into humin was proved by boiling freshly precipitated humic acids, obtained from peat and laevulose, with a 7·5 per cent. solution of hydrochloric acid for four hours, when it was found that 3·5 per cent. of the peat acid and 98 per cent. of the laevulose acid had been converted into humin.

The varying proportions of humic acid and humin in these deposits from sugar probably account for the diverse analytical results which have been obtained by various investigators.

A comparison of the so-called "artificial" humic acid from sugar with the "natural" product from soil or peat shows that in appearance, solubility and behaviour towards alkalies they are almost identical. Yet when analysed a considerable difference is seen to exist in their composition.

Robertson, Irvine and Dobson [1907] state that the average composition of different specimens of artificial and natural humic acids obtained by an ammonium salt was as follows:

	C	H	O
Artificial (sugar) humic acid	64·74	4·69	29·81
Natural (peat) „	54·29	4·94	38·16

This variation in composition leads these authors to consider that a structural difference exists between the artificial and natural acids.

Recent researches by Baumann [1909] have shown that natural humic acid freshly precipitated possesses colloidal properties, especially the power of forming adsorption compounds.

Considering that natural humic acid is formed in association with various decomposition products, it is probable that the true humic acid substances formed from the carbohydrates of the soil are admixed with various other products. As already indicated, some of these bodies have been extracted by means of alcohol from natural humic acid, and it was considered probable that, if the theory is correct that carbohydrates are the primary source of humic acid in the soil, the composition of the natural humic acid after purification by alcohol would approximate more closely to that of artificial humic acid.

This hypothesis was confirmed as follows: a specimen of "natural" humic acid was obtained by treating finely divided peat with a 4 % solution of hydrochloric acid until all soluble salts were extracted and then digesting the well-washed residue with an excess of 5 % ammonium hydrate solution. The dark brown liquid obtained after filtering the mass on a Buchner funnel was acidified with strong hydrochloric acid, and the flocculent brown precipitate thus obtained was separated by filtration and well washed. It was then redissolved in ammonia and reprecipitated by hydrochloric acid, the precipitate being washed with distilled water until free from chlorides. The residue was divided into two portions, one of which was dried in the steam-oven at 100°, while the other was boiled for one hour under a reflux condenser with absolute alcohol. The alcohol was filtered off, the residue washed with fresh alcohol, and dried. The average results of a series of combustion analyses of these two products were as follow:

	C	H	O (by diff.)
Natural humic acid	48·64	4·55	46·81
Natural humic acid after extraction with alcohol	60·37	5·39	34·24

These results indicate that when natural humic acid has been purified by means of alcohol its carbon content approximates much more closely to that of the artificial acid, the alcohol having removed most of the adsorbed bodies. It is well known that the carbon content of artificial humic acid varies with the method of its preparation, and no definite formula can be assigned to it, but it was found that the above analysis of purified natural humic acid showed a remarkable similarity with that of one of the artificial humic acids we obtained by boiling dextrose with hydrochloric acid. This is shown by the following comparison:

	C	H	O (by diff.)
Artificial humic acid	60·74	5·13	34·13
Natural humic acid (purified)	60·37	5·39	34·24

Humic Bodies from Sugars by the action of Organic Acids.

It is well known that the humic acid and humin groups of substances are formed during the decomposition of organic matter in the soil. It is also known that in the process of this decomposition various organic acids are produced by bacterial activities. Experiments were therefore made to determine the effect on sugars of certain organic acids known to exist in the soil.

It was found that lactic, acetic, propionic, butyric, citric, tartaric and oxalic acids when boiled with either sucrose, dextrose or laevulose yield humic acid and humin, after producing the characteristic series of colour changes obtained by the action of mineral acids on sugars. The rapidity of the action varies with the kind and concentration of acid used, as shown by the following tables:

The action of 12·5 % *acids on sucrose.*

Acid	Time for yellow colour	Time for red colour	Time for first deposit	Wt. of total ppt. in 24 hrs.	Wt. of re-ppd. humic acid in 24 hrs.
Lactic	1 hr. 15 mins.	2 hrs.	5 hrs. 10 mins.	0·123 gm.	0·082 gm.
Acetic	2 hrs.	7 hrs. 10 mins.	12 hrs. 10 mins.	0·050 gm.	0·025 gm.
Propionic	3 hrs.	9 hrs. 10 mins.	15 hrs. 30 mins.	0·016 gm.	0·009 gm.
Butyric	3 hrs. 10 mins.	12 hrs. 10 mins.	20 hrs.	0·024 gm.	0·004 gm.

The action of 36 % *acids on sucrose.*

Acid	Time for yellow colour	Time for red colour	Time for first deposit	Wt. of total ppt. in 24 hrs.	Wt. of re-ppd. humic acid in 24 hrs.
Lactic	Immediate	2 mins.	5 mins.	0·715 gm.	0·695 gm.
Acetic	5 mins.	35 mins.	10 hrs.	0·211 gm.	0·200 gm.
Propionic	Immediate	12 mins.	16 hrs.	0·365 gm.	0·164 gm.
Butyric	55 mins.	4 hrs. 40 mins.	18 hrs.	0·040 gm.	0·008 gm.
Citric	20 mins.	1 hr.	2 hrs. 40 mins.	0·042 gm.	0·024 gm.
Tartaric	10 mins.	15 mins.	30 mins.	0·009 gm.	0·009 gm.
Oxalic	Immediate	5 mins.	25 mins.	0·160 gm.	0·093 gm.

Humic Bodies from Sugars by the action of Heat.

In the course of the present investigation it was found that the action of heat alone on sucrose, dextrose and laevulose will produce bodies very similar to humic acid and humin.

It is well known that sucrose fuses at 160°, and is converted into a mixture of dextrose and laevulosan; on further heating to about 190° it yields caramelan and at higher temperatures gradually becomes carbonised.

In a preliminary experiment it was found that the black mass produced by heating sucrose at 220° for two hours contained at least four distinct substances: (1) water-soluble caramelan; (2) a water-soluble substance precipitated as fine particles by hydrochloric acid; (3) an alkali-soluble substance giving the typical flocculent precipitate of humic acid when treated with hydrochloric acid; (4) a black residue insoluble in water and alkalies giving the characteristic humin reactions when fused with caustic soda or potash.

Further experiments showed a great difference between dextrose and laevulose in their heat reactions, laevulose forming a black mass at a much lower temperature than dextrose. Dextrose on heating for four hours at 180° forms a light brown toffee-like mass completely soluble in water, but giving no precipitate with hydrochloric acid. On further heating for two hours at 220° it changes to a dark brown colour. Very little of this is water-soluble. The residue is almost entirely soluble in a 5 per cent. soda solution, and gives no trace of a precipitate with hydrochloric acid. On heating for three hours more at 280° it is converted into a black brittle mass, insoluble in both water and alkalies, but yielding humic acid on fusing with caustic soda and precipitating with hydrochloric acid. This black mass is evidently very similar to humin.

Laevulose even at the low temperature of 120° forms water-soluble substances which give a granular precipitate with hydrochloric acid if the heating be continued long enough, about six hours. When heated for two hours at 150° it gives a dark chocolate-brown mass all of which is soluble in water, giving a typical flocculent humic acid precipitate. Heated for two hours more at 180° it forms chiefly alkali-soluble humic acid, and further heating at 200° for two hours converts a large proportion of this into humin.

Here again, as in the acid-formed substances, humic acid is antecedent to humin. One important point of difference, however, must be noted between the acid-formed and heat-formed humic acid. The heat-formed humic acids after being dried in a water bath at 100° are no longer soluble in dilute alkali solutions; only on fusing with caustic potash or soda are they rendered soluble.

Humic Bodies from Proteins.

Proteins are said on hydrolysis to yield "an insoluble brownish black residue of humin." A careful examination of this black residue shows that it is chiefly composed of humic acid and contains very little humin. That

the formation of this humic acid depends on the amount of carbohydrate present in the protein is seen by comparing the amount of humic acid obtained from proteins with varying content of carbohydrate. Mucin and ordinary egg white are rich in carbohydrates; caseinogen and purified egg white are practically free from carbohydrates. One gram of each of these substances, the dry weight of the ordinary egg white being calculated by a moisture determination, was boiled for eight hours under a reflux condenser with 33 cc. of a 7·5 per cent. solution of hydrochloric acid. The contents of each flask were filtered, the residue extracted with a 5 per cent. solution of caustic soda, and the extract acidified with hydrochloric acid. The precipitate of humic acid was filtered, washed, dried and weighed, with the following results:

Mucin	0·021 g.	Caseinogen	0·001 g.
Ordinary egg white	0·035 g.	Purified egg white	no precipitate.

A similar experiment with tyrosine and asparagine gave no trace of humic acid after eight hours' boiling.

Conclusions.

The general result of the investigation so far has been to indicate that carbohydrates generally and certain sugars in particular pass through a regular series of changes when submitted to the reactions described. It was also found that air-dried *sphagnum* moss is readily acted on by acids, with the formation of a brown peat-like mass from which humic acid can be extracted.

One hundred grams of air-dried *sphagnum* moss when boiled for 24 hours with 5 per cent. solutions of hydrochloric, oxalic and lactic acids respectively yielded the following results:

Hydrochloric acid	11·7 g. humic acid
Oxalic acid	2·3 g. ,,
Lactic acid	1·8 g. ,,

The humic substances comprising the humic acid and humin groups probably pass through a series of changes characterised by a progressive increase of their carbon content. In ordinary cultivated soils these changes cannot be traced owing to the constant addition of fresh organic matter, and cultural operations, but in peat beds, where the deposits remain undisturbed for many years, the stages are indicated by the increasing carbon content of the humus from varying depths. This was shown by Detmer [1871], who gives the following figures:

	C	H	O
Light brown peat, surface	52·14	7·03	40·19
Brown peat, 1 foot	57·75	5·43	36·02
Dark peat, 7 feet	62·02	5·21	30·67
Black peat, 14 feet	64·07	5·01	26·87

Thus in peat bogs the carbohydrates of decaying organic matter may possibly pass through changes very similar to those observed when sugar is heated to a high temperature: sugar, caramelan, humic acid, humin, and finally carbonisation (peat coal). The fact established by Van Bemmelen [1900] that humic acid is a colloid body which has the property of uniting with certain radicles explains the various empirical formulae ascribed to humic acid, and the complex nature of humus in general. But underlying this complexity there is the possibility of finding in the series of sugar changes described above two fairly definite groups of substances which serve as a basis for humus formation, the humic group gradually merging into the humin group.

Summary.

1. Sugars on boiling with hydrochloric acid yield a mixture of humic acid and humin bodies, varying in proportion with the different sugars used.

2. The composition of "natural" humic acid from soil or peat, after purification by alcohol, approximates very closely to that of "artificial" (sugar) humic acid.

3. Humic acid and humin are also produced from sugars by the action of various organic acids: lactic, acetic, propionic, butyric, etc.

4. The action of heat alone on sugars produces humic acid and humin bodies, these substances being stages in the process of carbonisation.

5. Humic bodies are not obtainable from proteins free from carbohydrates.

6. The two groups of humic bodies—humic acid and humin—obtained artificially from carbohydrates, indicate a basis for the natural processes of humus formation.

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XXIV. THE FREE AMINO NITROGEN OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM.

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In a recent communication van Slyke and Birchard [1913] have shown that when certain native proteins are treated with nitrous acid the amount of nitrogen formed as a result of the reaction is equal to one-half the lysine nitrogen of the protein. They suggest that one of the amino groups of lysine, the ω-group, exists free in the intact protein molecule, and, moreover, that this ω-amino nitrogen of the lysine represents practically the entire amount of free amino nitrogen which can be demonstrated by the nitrous acid method of van Slyke. All the amino acids, except lysine, react quantitatively with nitrous acid in three to four minutes; lysine reacts more slowly, thirty minutes being required for complete reaction, and van Slyke and Birchard show that reaction between nitrous acid and the native proteins also requires about thirty minutes to attain completion. The authors quote observations of other workers which support their view that the free amino nitrogen of native proteins is due to the ω-amino group of the lysine which they contain. Gelatin, caseinogen and serum globulin after treatment with nitrous acid yield no lysine on subsequent hydrolysis with acid. Clupeine, which contains no lysine, yields no nitrogen on treatment with nitrous acid, while sturine and cyprinine, which contain lysine, do. It has also been shown that certain protamines and proteins which contain lysine reveal free amino groups by the formal titration method of Sörensen, while those which contain no lysine do not.

The analysis of the different proteins of ox and horse serum, an account of which has recently appeared [Hartley, 1914], shows that albumin differs in several important respects from the globulins. In particular it has been found that albumin contains a much larger amount of lysine than the globulins. The experiments described in this paper have been carried out in order to study the relationship between the free amino nitrogen of the different serum proteins and their lysine content.

The preparation of the different serum proteins has been described in the earlier paper. For the determination of the amino nitrogen the following procedure was adopted in each case. Two weighed quantities of the air-dried preparations were taken: one of these was used for the determination of the total nitrogen of the protein by Kjeldahl's method and the other was dissolved in water or very dilute alkali in a standard 15 cc. flask. Ten cc. of this solution were treated with nitrous acid in van Slyke's apparatus and the free amino nitrogen of the protein determined. The reaction was conducted in the standard size apparatus described by van Slyke [1912], 1 cc. of octyl alcohol being added in each case to prevent frothing. The mixture of nitrous acid and protein was shaken at intervals for twenty-five minutes and finally shaken continuously for five minutes.

The results of the determination of the total nitrogen and the free amino nitrogen are collected in the following tables.

Total globulin is the name given to the proteins precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudoglobulin and euglobulin. Euglobulin *A* was prepared from the total globulin by salting out with ammonium sulphate, and euglobulin *P* was prepared by the dilution and acidification method of Panum.

TABLE I.

Total Nitrogen Determinations.

No.	Protein	Quantity taken g.	N/10 H_2SO_4 neutralised cc.	Nitrogen mgm.
1.	Albumin (ox)	0·2065	18·8	26·32
2.	Total globulin (ox)	0·2110	19·9	27·86
3.	Albumin (horse)	0·2562	23·7	33·18
4.	Total globulin (horse)	0·2604	24·9	34·86
5.	Pseudoglobulin (horse) ...	0·2882	27·1	37·94
6.	Euglobulin *A* (horse)	0·2894	26·4	36·96
7.	Euglobulin *P* (horse)	0·3047	25·2	35·28
8.	Whole proteins (horse) ...	0·2761	23·3	32·62

TABLE II.

Amino Nitrogen Determinations.

No.	Protein	Quantity taken gm.	N gas cc.	Temp. °C.	Pressure mm.	Amino N mgm.	Per cent. of total N as free NH_2
1.	Albumin (ox)	0·5157	9·9	16	755	5·70	8·7
2.	Total globulin (ox) ..	0·5013	4·8	16	758	2·80	4·2
3.	Albumin (horse)	0·3875	7·0	17	762	4·05	8·0
4.	Total globulin (horse)	0·3932	5·2	19	762	2·98	5·6
5.	Pseudoglobulin (horse)	0·2806	3·1	15	760	1·81	4·6
6.	Euglobulin *A* (horse)	0·4096	5·4	17	749	3·07	5·8
7.	Euglobulin *P* (horse)	0·4050	4·3	17	749	2·44	5·2
8.	Whole protein (horse)	0·3963	5·1	19	762	2·92	6·2

In Table III the results of the determination of the free amino nitrogen are given and compared with the lysine content of the different proteins. The figures for the lysine are taken from the earlier paper [1914] dealing with the analysis of the different proteins of ox and horse serum.

TABLE III.

No.	Protein	Per cent. of total nitrogen as Free amino N	Half lysine N
1.	Albumin (ox)	8·7	8·15
2.	Total globulin (ox)	4·2	4·50
3.	Albumin (horse)	8·0	7·95
4.	Total globulin (horse) ...	5·6	5·40
5.	Pseudoglobulin (horse) ...	4·6	4·90
6.	Euglobulin *A* (horse)	5·8	4·97
7.	Euglobulin *P* (horse)	5·2	5·05
8.	Whole proteins (horse) ..	6·2	6·40

Except in the case of Euglobulin *A* (horse) in which the result is a little high, the free amino nitrogen of the proteins of ox and horse serum is equal to one-half the lysine nitrogen. The results are in agreement with those obtained by van Slyke and Birchard for other native proteins and lend support to the suggestion advanced by them that in the intact protein molecule the ω-amino group of the lysine exists free, and, further, that this group represents practically the whole of the free amino nitrogen determinable by means of nitrous acid.

The results given above, taken in conjunction with those of van Slyke and Birchard, indicate that the lysine content of a native protein may be rapidly estimated by determining the amino nitrogen and the total nitrogen of the protein.

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XXV. SOIL ORGANIC MATTER AS A CULTURE MEDIUM FOR *AZOTOBACTER*.

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Not the least important of the functions performed by soil organic matter is that of providing food material for the bacterial flora upon the activity of which the fertility of the soil so largely depends. The problem of maintaining soil fertility often resolves itself into a question of promoting bacterial activity, and it is safe to say that the most important of all the soil organisms are those concerned in the nitrogen cycle. The nitrifying organisms assimilate the carbon dioxide of the air, and are thus independent of organic carbon; the ammonifying organisms can do their work when provided with the humus, or any organic matter containing nitrogen, so long as potassium salts are present [Dumont, 1905]; but the nitrogen fixing organisms, and especially *Azotobacter*, are generally supposed to be somewhat sensitive in their requirements, flourishing only upon specialised non-nitrogenous organic compounds. A wide variety of such substances pass into the soil from the natural accumulations of humus by the ploughing in of green crops and in the application of rotted farm and stable manure. These compounds undergo decomposition in the soil through the agency of many micro-organisms, including the spores and mycelia of the higher fungi [C. van Iterson, 1904]. The various enzymes which are introduced with the organic matter remain active in the soil [Woods, 1899], and assist in the further decomposition of these compounds; while the intact roots of plants have the power of oxidising the organic matter and bringing about an appreciable change in its composition [Molisch, 1887; Schreiner and Reed, 1909]. The higher organisms

such as worms are active in splitting up the fats and other complex substances; while in addition to these organic agencies at work, there must be taken into account the purely chemical changes which are constantly taking place. The nett result of all these reactions is the production of a large number of organic compounds, both nitrogenous and non-nitrogenous; the latter ranging in complexity from carbohydrates down to the simplest alcohols. It is generally assumed that carbohydrate in some form is essential for the nutrition of *Azotobacter*, although cultures have been obtained upon certain other substances, notably some of the vegetable acids, and the object of the present investigation was to discover how far the organism is capable of utilising the wide range of non-nitrogenous organic compounds which occur in the soil.

Method of work.

A uniform method of procedure was adopted throughout the work. The culture medium employed, with variation of the source of organic carbon only, was that used by Bottomley and given in detail in a previous communication by the writer [Mockeridge, 1912]. For the determination of the nitrogen fixing power of the organism upon each substance, 50 cc. portions of the medium, containing 1 % of the nutrient, were placed in each of six 300 cc. Erlenmeyer flasks, and sterilised in the autoclave at a temperature of 140°. Where organic acids were employed, sufficient calcium carbonate to neutralise them was added to each flask, and in the case of volatile organic substances, these were not added to the medium until the flasks containing the inorganic salts in solution had cooled down after sterilisation. Each flask was provided with a sand slope consisting of 50 grams of pure sterile sand, and was then inoculated with 1 cc. of a uniform suspension in distilled water of *Azotobacter* from a mannitol-agar plate. Two flasks of each series were then again sterilised to serve as controls, and the whole series was incubated at a temperature of 26° until all trace of the organic substance originally supplied had disappeared from the medium, when the contents of each flask were analysed by the Kjeldahl process for its nitrogen content.

Humates.

It is doubtful whether soluble humates can serve as a source of food material for *Azotobacter*, although it has been proved [Krzemieniewski, 1908] that they stimulate the activity of the organism, when the latter is already

provided with another nutrient; and if this takes place in artificial media and under the abnormal conditions of the laboratory tests, the probability is that it occurs more markedly still in the soil. In the present work it was found possible to obtain a growth of *Azotobacter* upon a solution of ammonium humate containing the requisite mineral salts, although no increase in the nitrogen content of the medium took place; but such growth could not be obtained upon media containing potassium or calcium humate without any other source of organic carbon. The organism thus appears to be able to utilise the humates as a source of energy if provided with nitrogen already fixed, and ammonium humate is present in all fertile soils in small, though appreciable, quantities; the ammonia produced by the decomposition and ammonifying organisms uniting with the humic acid in the organic matter.

Polysaccharides.

The widespread occurrence of various gums and starches in vegetable tissues is well known, and these substances must be transferred to the soil in all accumulations of plant débris. The value of dextrins as a source of carbon for mixed cultures of nitrogen-fixing organisms has already been pointed out [Bottomley, 1912]; and since the enzyme (amylase) directly concerned in their production is practically always found in foliage leaves [Brown and Morris, 1893], the addition of these bodies to the soil in green manures, as well as their production in the soil itself and in rotting manure-heaps, by hydrolysis of cellulose and starch are matters of certainty. Inulin is also a common food reserve in plants. As therefore the various polysaccharides must naturally occur in soils, an examination was made of the availability of certain of them as nutrients for *Azotobacter*. Gum arabic, gum tragacanth, starch, dextrin and inulin were tested, and all proved to be readily available for the organism.

The fixations obtained were as follows:

TABLE I.

Substance	Nitrogen fixed on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram of the substance. Days
Gum arabic	6·13	60
Gum tragacanth .	9·13	20
Rice starch	6·40	18
Potato starch ...	5·93	20
Dextrin	6·62	20
Inulin	9·76	16

Sugars.

Sugar in some form is the usual nutrient supplied to *Azotobacter* in artificial cultures, and the power of the organism to utilise most of the more readily obtainable sugars was tested. These included pentoses and hexoses among the monosaccharides, and some of the disaccharides.

The results obtained were as follows:

TABLE II.

Sugar	Nitrogen fixation on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram. Days
Arabinose	9·28	30
Xylose	9·00	28
Dextrose	6·57	20
Laevulose.....	10·32	18
Galactose	6·20	22
Sucrose	7·28	22
Maltose	7·55	17
Lactose	3·39	34

The fact that the organism is capable of utilising two of the pentoses, arabinose and xylose, renders it probable that other members of the group, which occur somewhat widely in nature, are also available; for pentose sugars are formed both in the soil and in the manure-heap by the hydrolysing action of certain bacteria and enzymes upon gums, pectin compounds, naturally occurring glucosides, nucleic acids, etc. Recently a pentosan of undetermined nature, and also rhamnose itself, have been isolated from samples of loam [Schreiner and Shorey, 1910; Shorey, 1913]. Similarly the wide distribution of the hexoses and disaccharides in nature, and the variety of the agencies by which they may be produced in all decomposing vegetable matter, indicate a wide range of available food for *Azotobacter* in the soil.

Alcohols.

Among the substances tested as a source of food for *Azotobacter* were some of the monohydric alcohols, besides representatives of some of the more complex alcoholic compounds, as ethylene glycol (a dihydric alcohol), glycerol (trihydric), erythritol (tetrahydric) and mannitol (hexahydric). The fact that substances of an alcoholic nature are readily assimilable by *Azotobacter* is shown in the following table:

TABLE III.

Alcohol	Nitrogen fixed on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram. Days
Methyl alcohol ..	2·1	42
Ethyl alcohol ...	4·02	34
Propyl alcohol ..	9·2	22
Isobutyl alcohol .	4·69	72
Ethylene glycol .	16·74	34
Glycerol	5·0	56
Erythritol	4·88	60
Mannitol	11·62	15

Only one determination of the nitrogen fixed upon ethylene glycol was made, on account of the difficulty of obtaining larger quantities of the compound; but a very copious growth of the organism upon this nutrient was obtained.

Flasks containing amyl alcohol and inoculated with *Azotobacter* showed no growth of the organism at the end of a month, but upon a solid medium obtained by the use of agar-agar a fair growth appeared upon this nutrient. No determinations were made of the nitrogen fixed.

The alcohols are of very frequent occurrence in nature, and appear to be sufficiently abundant in soil organic matter to warrant their inclusion among the bacterial foodstuffs of the humus. The possible sources in the soil of the alcohols mentioned in the above table are indicated below. Methyl alcohol occurs in combination in various plants, and is produced in the free state in cow dung by the action of *B. boöcopricus*. Ethyl alcohol is a well-known product of yeast fermentations and of practically all bacterial action, while propyl alcohol usually accompanies it in small quantities as a by-product. Butyl alcohol is formed during butyric fermentation, while under certain conditions *B. amylozyma* will produce amyl alcohol. Ethylene glycol is very probably a by-product of bacterial action in the soil, for one of its higher homologues, butylene glycol, has been shown to be formed during the action of *B. lactis aerogenes* on glucose and mannitol [Harden and Walpole, 1906]. Glycerol is a well-known by-product in yeast fermentations and in many bacterial actions; erythritol is found in the free state in lichens, mosses and seaweeds, while mannitol occurs naturally in many plants. The last-named substance has been isolated in fairly large quantities from one soil [Shorey, 1913].

Hence it is probable that substances of an alcoholic nature occur fairly widely in the soil, and are sufficiently abundant to provide a convenient source of food for *Azotobacter*.

Vegetable acids (other than aliphatic).

The organic acids are constant products of decomposition of practically all organic matter, and so probably most of them occur in soils and manures. The nitrogen-fixing power of *Azotobacter* when grown upon a medium containing the calcium salts of the organic acids was tested, with the following results:

TABLE IV.

Acid	Nitrogen fixed upon 1 gram (average of 4 determinations) Mg.	Time taken to use completely 1 gram. Days
Citric	6·44	48
Malic	5·19	16
Tartaric	4·54	28
Racemic ...	2·77	50
Succinic	8·60	20
Malonic	5·32	20
Mucic	6·79	40
Fumaric ...	2·00	50
Maleic	1·88	50
Glycollic ...	1·75	60
Lactic	12·01	17

The vegetable acids are widely distributed in nature, citric acid, malic acid and tartaric acid and its stereo-isomers being among the most commonly occurring; the first-named, besides its occurrence in the free state, arises also as a product of the action of various micro-organisms. Succinic acid is also found in many plants, besides which it is a product of putrefaction and occurs in the urine of goats, horses, rabbits and cows, so that it is probably transferred to the soil in rotting manure, in which is also to be found lactic acid—a product of the lactic fermentation of carbohydrates in the presence of nitrogenous animal matter. The remaining acids tested, mucic, glycollic, maleic, fumaric and malonic, though they do not occur in plant residues, so far as is known at present, may readily be produced in soil or manure from the other acids enumerated, or from other organic substances by the various agencies at work during the decomposition of soil organic matter. That mucic acid, for instance, probably exists in the soil is indicated by the fact that saccharic acid, an isomeric form, has actually been isolated [Shorey, 1913], although this is not known to occur in plants. From the same soil were isolated oxalic, succinic and acrylic acids, while from other samples paraffinic, lignoceric, α-hydroxystearic and some resin acids have been isolated [Schreiner and Shorey, 1910], while still other soils have been found to contain dihydroxystearic, picolinecarboxylic and agroceric acids [Schreiner and Shorey, 1909].

Meconic acid was found to be useless as food for *Azotobacter*, but the substance itself is not inhibitory to the organism, for good cultures were obtained upon mannitol-agar plates containing meconic acid. Probably some of its products of decomposition have a deleterious effect. The poisonous nature of oxalic acid accounts for the fact that it is not available as a source of food for the organism.

Organic acids of any kind are useless to *Azotobacter* until neutralised by lime or some other base, but this presents no difficulty, since there is abundance of lime for the purpose in all fertile soils.

Fatty acids.

In the series of aliphatic acids, those tested with regard to their availability for *Azotobacter* included formic, acetic, propionic, butyric, isovaleric, stearic and palmitic acids. On the last three no growth was obtained in liquid culture, but when the medium was solidified by the addition of 2 % agar-agar, and poured into Petri dishes, a fairly abundant growth was obtained upon the surface. The organism is thus capable of utilising these substances as a source of energy, although it requires special conditions, probably connected with air supply. No determination of the nitrogen fixed upon these substances was made. Formic, acetic, propionic and butyric acids formed readily available sources of food, and the figures obtained upon these media are given in greater detail than has hitherto been the case, since further reference will be made later to these results.

The fatty acids are very widely distributed in nature. The more complex fats and oils are of almost universal occurrence in the vegetable kingdom, being found as reserve stores in fruits and seeds. Oil cakes of various kinds form a large part of the diet of animals at fattening time, so that an appreciable quantity of undigested fat and oil is probably transferred to the manure heap. These complex bodies of a fatty nature undergo decomposition through bacterial action under certain conditions of light and aeration with production of the simpler aliphatic acids. Reserves of fat in plants are always accompanied by the fat splitting enzymes, so that in the soil the decomposition of the fat into glycerol and a fatty acid, most commonly oleic, palmitic and stearic, may proceed. The lower aliphatic acids are also distributed in fair quantity throughout rotting manures, being formed by the action of bacteria upon many substances, especially upon the degradation products of the proteins; so that members of this group, when

neutralised by lime or some other base, serve as a readily available source of food for *Azotobacter*.

TABLE V.

Nature of medium	Nitrogen content, mg.	Fixation on ½ g., mg.	Fixation on 1 g., mg.	Mean Fixation, mg.	Time taken to use 1 g.
50 cc. 1 % formic acid control	0·29				
	0·29				
„ „ with *Azotobacter*	0·91	0·62	1·24		
„ „ „	1·22	0·93	1·86	1·47	60 days
„ „ „	1·06	0·77	1·54		
„ „ „	0·91	0·62	1·24		
50 cc. 1 % acetic acid control	0·16				
	0·16				
„ „ with *Azotobacter*	2·26	2·10	4·20		
„ „ „	2·12	1·96	3·92	3·77	24 days
„ „ „	1·98	1·82	3·64		
„ „ „	1·83	1·67	3·34		
50 cc. 1 % propionic acid control	0·41				
	0·41				
„ „ with *Azotobacter*	3·14	2·72	5·44		
„ „ „	2·86	2·44	4·88	5·16	26 days
„ „ „	3·00	2·58	5·16		
„ „ „	3·00	2·58	5·16		
50 cc. 1 % butyric acid control	0·14				
	0·14				
„ „ with *Azotobacter*	3·18	3·04	6·08		
„ „ „	3·04	2·90	5·80	6·08	48 days
„ „ „	3·32	3·18	6·36		
„ „ „	3·19	3·05	6·10		

Esters.

The ethereal salts, or esters, occur widely, although in small quantity, in the essences of flowers and fruits and certain leaves. They probably find their way into the soil from these sources, and there undergo decomposition, through the agency of the lipases or other hydrolysing agents, into their two primary constituents; but even in the free state they are able to serve as a source of energy for *Azotobacter*. Two only of the simple esters were tested: ethyl and butyl acetates, and upon neither of these in liquid culture could any growth be obtained. Upon agar plates containing these substances, however, a fairly abundant, though slow, growth took place.

Glucosides.

The glucosides are of very frequent occurrence in plant substances, but their availability as food for this nitrogen-fixing organism appears to be restricted somewhat by the products of their decomposition. An abundant

growth, but a very small fixation of nitrogen, was obtained upon a solution of amygdalin, a glucoside occurring in bitter almonds and the kernels of several stone fruits. This glucoside upon hydrolysis gives rise to glucose, benzaldehyde and hydrocyanic acid, and these are the products of the action of yeast upon it. The glucose, of course, is immediately available for *Azotobacter*, and neither the hydrocyanic acid nor the benzaldehyde appears to be inhibitory to its growth. Hydrocyanic acid is found extensively in the free state in plants, so that small quantities of this substance are evidently not deleterious to plant life.

Most of the naturally occurring glucosides, e.g. salicin, give an alcohol upon hydrolysis, and salicin was found to be useless for the growth of *Azotobacter*, either in liquid or solid media. This fact points to the conclusion either that the organism has no action on the glucoside or that its first action is a hydrolytic one; for salicin, upon hydrolysis, gives glucose and salicyl alcohol, which latter is a powerful antiseptic, and would accordingly inhibit the growth of the organism. The substance itself is not harmful to *Azotobacter*, for an abundant growth of the organism was obtained on mannitol-agar plates which contained salicin; a fact which indicates that it must be the decomposition products of the salicin which have a deleterious effect.

Benzene derivatives.

A similar state of affairs was found to obtain with all the benzene derivatives tested. These included phenylacetic acid, mandelic (phenylglycollic) acid, benzyl alcohol, pyrogallol, quinol and catechol. These substances will not serve as a food supply for the organism in either liquid or solid media, yet when mixed into mannitol-agar plates do not inhibit its growth. Such substances as benzyl alcohol, catechol, pyrogallol and quinol are not readily split up chemically, and the great stability of the benzene ring may account for the unsuitability of these substances as food for *Azotobacter*; moreover, from such substances as phenylacetic and phenylglycollic acids there is always the possibility of the formation of phenol, and this product might prevent any further bacterial growth. The inability of the organism to utilise substances of this nature thus apparently depends not upon any harmful effect produced by the compounds themselves, but either upon the injurious secondary products formed by their decomposition, or on the difficulty of breaking up the stable benzene ring.

General considerations.

The results obtained indicate the wide range of substances which are available as a source of energy for this nitrogen-fixing organism in the soil: substances which occur naturally and may be added to the soil in green manures or in accumulations of vegetable débris; compounds which may be produced by fermentation of rotting manures and added to the soil in that way; and products which arise by secondary and often purely chemical reactions in the soil itself. It is of interest to note also, that the large number of organic substances present in the soil, which the organism cannot utilise, do not necessarily inhibit its growth, so long as some available food material is present; for *Azotobacter* appears to be possessed of considerable selective power.

All classes of compounds do not give equally good returns of nitrogen fixed for the expenditure of food material, and, generally speaking, it seems to be the rule that the longer the time taken to use completely unit mass of the nutrient, the less the nitrogen fixed upon that medium. Though they serve as a general guide, too much reliance must not be placed upon the figures obtained in this respect, for, as the author has pointed out in previous work, the nitrogen-fixing power of the organism varies considerably with the length of time during which it has been grown upon artificial media.

In order to compare correctly the relative availability of a large number of nutrient substances, the same strain of *Azotobacter* should be used throughout for inoculating purposes, and this means that all the cultures required should be incubated at once, whereas in practice the work has to be carried out in sections.

Generally speaking, the carbohydrates give the greatest yield of nitrogen fixed per unit of material consumed, and it is only to be expected that in the breaking down of the more complex molecule more energy should be obtained by the organism, and thus that more growth and consequent fixation of nitrogen should take place. *Azotobacter* is essentially an aerobic organism, and Beijerinck and van Delden [1902] observed that it absorbed oxygen and eliminated carbon dioxide, their observations being confirmed by the careful work of Krzemieniewski [1908]. This points to a slow combustion of the organic material provided; and some idea of the energy thus obtained by the organism can be gained by a calculation of the heat of combustion of the carbon compound supplied. When these figures are calculated for the aliphatic acids and compared with the amounts of nitrogen fixed upon those substances as given in Table V, the following results are obtained:

TABLE VI.

Acid	Molecular weight	Heat of combust. of 1 gram-molecule	Heat of combust. of 1 gr.	N fixed on 1 gr.	Ratio N fixed : Heat of combust.
Formic	46	68300 cals.	1484 cals.	1·47 mg.	1 : 1009
Acetic	60	230900	3848	3·77	1 : 1020
Propionic ...	74	393500	5317	5·15	1 : 1032
Butyric	88	556100	6319	6·08	1 : 1039

Within the range of experimental error, these ratios may be taken to be approximately constant, a result which points to the conclusion that in these four media, the decomposition of the respective organic constituents follows the same course, a very definite proportion of the energy thus obtained being devoted to the fixation of nitrogen.

It is obvious that, on account of the varied nature of the possible products of decomposition, such comparisons can only be made between substances of similar constitution; but the figures obtained for some of the carbohydrates are of interest.

TABLE VII.

Carbohydrate	Heat of combustion of 1 gram	Fixation on 1 gram	Ratio N fixed. Heat of combustion
Starch ...	4145 cals.	6·40 mg.	1 : 647
Dextrin ..	4145	6·62	1 : 626
Gum arabic	4145	6·13	1 : 676
Inulin	4145	9·76	1 : 424

Allowing for error of experiment, which is considerably greater here than in the case of the simpler compounds, owing to the difficulty of ascertaining that all the secondary products of the carbohydrate have completely disappeared, the ratios for starch, dextrin and gum arabic may be taken to be approximately equal. Assuming that hydrolysis takes place, as it must do, the starch will be converted finally into dextrose, with dextrin and maltose as intermediate products. A comparison of the ratios obtained with these substances is found to be

Starch	→	Dextrin	→	Maltose	→	Dextrose
1 : 647		1 : 626		1 : 598		1 : 585

The ascending order of these ratios bears out the assumption that a hydrolysis of the polyoses takes place to form the simple hexoses, which latter are then oxidised. The ratio obtained for inulin (1 : 424) is surprisingly high as compared with those for starch, dextrin and gum arabic. However, inulin upon hydrolysis gives laevulose, and the ratio between the nitrogen fixed on one gram of laevulose and the heat of combustion of one gram of the same body is found to be 1 : 372, which compares very well with the inulin ratio.

The close relation which seems to exist in the series of aliphatic acids between the nitrogen fixed and the nutrient consumed is not apparent in all classes of homologous compounds, for the series of monohydric alcohols shows no such relation; and some of the simpler compounds, as ethylene glycol, show a surprisingly high rate of fixation of nitrogen for the heat-energy obtainable. However, nothing is known as to the actual mechanism of fixation. It may be that the nitrogen is attached to very simple organic compounds produced by the splitting up of the nutrient, and that certain substances give decomposition products which are peculiarly adapted to the purpose, while the secondary products from others may be practically useless. On the other hand, a synthesis may take place between the simpler bodies to form substances more complex, before the nitrogen is attached, certain nutrients furnishing the necessary synthetic products with less waste of energy and material than others. That synthesis of non-nitrogenous substances takes place in the cells of *Azotobacter*, apart from nitrogen assimilation, is shown by the fact that appreciable quantities of fat have been found stored up in the cells [Lipman, 1904]. By no means the whole of the energy obtainable from the nutrient is utilised in nitrogen fixation, for the author above quoted states that according to thermo-chemical calculations, one gram of mannitol would allow of the fixation of 97·3 mg. of nitrogen. However, the few data obtained appear sufficient to warrant the conclusion that a detailed study of energy considerations may help to throw some light upon the manner in which the nitrogen is fixed; but no matter what course this may follow, it is evident that any ordinary fertile soil contains abundant food material for the growth of *Azotobacter*.

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XXVI. NOTES ON COLLODION MEMBRANES FOR ULTRAFILTRATION AND PRESSURE DIALYSIS.

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The differential porosity of gel-membranes to molecules of different sizes may be utilised in a number of different ways all of which are essentially filtration. If the "filtrate" side of the membrane is bathed in any solution other than filtrate the process may be called "dialysis." Filtration under pressure is a very common procedure: but the many advantages to be obtained by dialysis under pressure have not received the study and recognition they undoubtedly deserve.

The difference between ultrafiltration and pressure dialysis.

Pressure dialysis brings about the complete removal of any constituent of the material handled whose molecules are by reason of their size and nature capable of traversing the membrane. Ultrafiltration, on the other hand, permits only the removal of those small molecules which can actually be brought into contact with the filter and pushed through. Hence, in order to separate a mixture of materials into those constituents whose molecules pass through a certain diaphragm and those whose molecules do not, either ultrafiltration or pressure dialysis may be employed. The latter process may be regarded as that which gives a quantitative separation, with the disadvantage that the materials passing through the membrane are recovered in a dilute condition, although the volume of residual solution is completely under control.

Collodion membranes. The use of membranes of collodion for the processes referred to will always be associated with the names of Rodewald and Kattein [1900], Malfitano [1904], Duclaux [1905, 1907], Moore and Roaf [1907, 1908], Lillie [1907] and Bechold [1907, 1, 2, 3; 1908]. The last author used pressure and introduced the use of formalised gelatin as a filtering medium. To him, too, we are indebted for the word "ultrafiltration," which has now passed into general use. In this matter C. J. Martin [1896] anticipated many later developments by fully describing and illustrating special apparatus by which solutions to be investigated were forced under pressure through films of gelatin or silicic acid. The material impregnated was a Pasteur-Chamberland filter candle, which served as a support against disruption. In conjunction with T. Cherry [1898] such filtrations were used to demonstrate that diphtheria toxin-antitoxin mixtures after two hours' standing at 30° gave toxin-free filtrates, although toxin when alone passed through the filters, and antitoxin, as shown by Brodie [1897] using Martin's method, did not.

My object in this paper is to draw attention to the possibility of standardising accurately a series of filters, suitable for "ultrafiltration" and pressure dialysis operations, which are already in use. Also, to call attention to certain members of the series, that is to say those containing 5 mg. of nitrocotton per sq. cm. and weighing 17·5 mg. per sq. cm. because of contained water. These have been discovered to be impermeable to all antigens tried, although they allow water, salts and simpler molecules to pass freely—a valuable conjunction of properties not shared, as far as I can discover, by the ultrafilters in general use.

To make the matter clear, it may be stated as a result of direct comparative experiments with parchment and from experience in the past with gelatin filters, that attempts to force diphtheria toxin under pressure of two atmospheres through parchment or gelatin would not result in nearly so much filtrate as if one of the "antigen-proof" collodion films to which I have referred were used. Further, as may be seen by referring to the original papers from the references given, they would not be found to keep back antigen to any great extent.

The reason of this must, of course, in the present state of our knowledge of gel structure and the phenomena of filtration generally, be given with reservation. It would appear, however, not an unreasonable suggestion that these films possess an enhanced uniformity of structure and a great porosity. In other words, the channels leading from one side of the filter

to the other are none of them sufficiently large to let the antigen pass, but there are many of them. On the other hand, it is suggested that in parchment, for example, these holes are of irregular size. Some are large enough to let diphtheria toxin through, but the total amount of water they will allow to pass is, in virtue of the lower general porosity of the structure, less in this case [Glenny and Walpole, 1915].

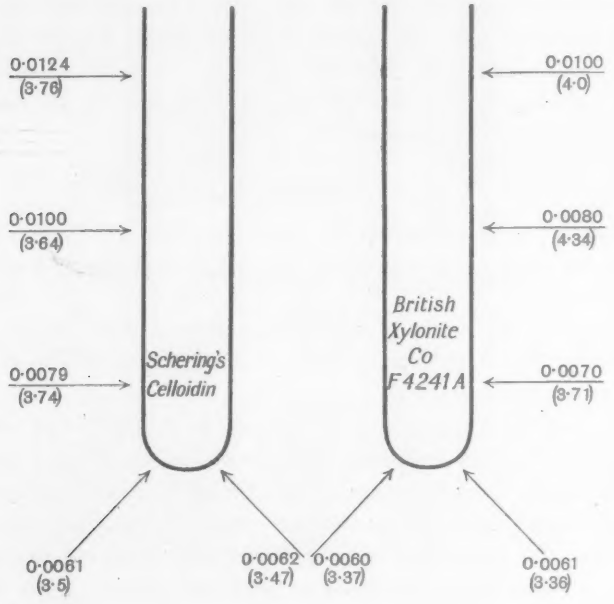
Collodion used. As a stock solution a 14 per cent. solution by weight of Schering's celloidin in equal parts of alcohol and ether was originally employed. Later the British Xylonite Company have kindly supplied me with a similar material ready dissolved and filtered clear. It is named for reference F 4241 A and supplied by them in tins with convenient screwed stoppers at a price which represents a considerable saving in expense. The solutions when used in the same manner give very similar results as may be judged from the comparative experiments given below (page 287).

The preparation of collodion bags. These have been often described by Malfitano [1904], Bigelow [1907] and many other authors. That they may be made with differences of porosity is generally known. For information about them and instructions how to make them I am indebted to Prof. S. P. L. Sörensen and Dr Christiansen. They are sometimes made by pouring consecutive layers of the solution on to the outside of a test tube rotated by a suitable mechanism. Probably the bags made in this way are more perfect than those made inside test tubes, but special apparatus is required and, I should judge, considerable experience and skill in order to be sure of producing a bag of just the required impermeability. The following is a description of the bags which I have adopted as "standard," though for anything beyond odd quantitative experiments I prefer to use the flat sheets described in some detail later, for they possess certain advantages over any cylindrical bag however perfect.

A test tube is filled with "stock solution," capped with tin foil and after the twelve or fifteen hours required for the bubbles to rise is inverted and clamped so that the mouth is well above the wide mouthed stock bottle. When the flow of collodion ceases the bottle is removed and stoppered and about ten minutes later the drippings are cut away from the test tube with a sharp knife, care being taken not to pull loose the lower edge of its collodion lining. The test tube must, of course, be cut off sharp and not lipped or opened at the mouth in any way. Drying is allowed to continue until the inside when rubbed gently with the finger does not mark at all—a degree which cannot be described precisely—a process taking about half an hour at

laboratory temperature. The test tube and its invisible collodion lining are now placed under water, and at a later time while still under water the lining may be easily removed and tied to a rubber stopper. It is convenient to remember that these membranes shrink rapidly in air and become less porous, though in cold water they may be stored indefinitely. In hot water they shrink, but may be stretched and moulded without undergoing any apparent permanent change—a property which makes it possible to mount them on rubber stoppers of slightly larger diameter than would otherwise be possible.

Some simple measurements of two bags, made at the same time, one from Schering's celloidin (13·45 per cent. by weight in equal parts dry alcohol and ether) and the other from British Xylonite Company material, F 4241 A (14·66 per cent. in the same solvent), may be of interest. The unavoidable lack of uniformity of bags made in this way is apparent from the diagram below, in which the figures represent the weight per sq. cm. of dry nitrocotton and the "wetness" at the points indicated (see page 292). The latter are in brackets. The dimensions of these bags are 4·5 cm. wide and 22 cm. long.



Smaller bags than this, even though made with great care, are still less uniform. Three other bags from each material, made at the same time under conditions as similar as it was possible to arrange, were tested. One pair filtered diphtheria toxin, a second pair filtered tetanus toxin, while a third pair were tested by pressure. They both ruptured between two and three atmospheres pressure after stretching considerably.

Ultrafiltration of tetanus toxin. Batch Z. Both bags were fed with toxin at 20 cm. of mercury pressure for about a week; the Schering's celloidin bag passed 2600 cc., while that made from the British Xylonite Company's material passed 2300 cc. The former filtrate gave a very faint indication of toxicity: none could be detected in the latter.

Ultrafiltration of diphtheria toxin (mixture of equal parts J 2133 and J 2159, L_+ dose 0·40 cc.: nitrogen = 5·3 mg. per cc.). The pressure applied equally to both bags was 20 cm. of mercury for twelve days. The bag from Schering's celloidin allowed 1875 cc. to pass; that from British Xylonite Company material filtered 2155 cc. No toxin could be detected in the latter filtrate: 1 cc. of the former gave a slight swelling in the guinea pig.

It may be remarked in passing that the residues in the bags were, at this juncture, surrounded by 0·3 per cent. phenol changed daily for three days and then examined. The volumes were 100, 85 cc.; the nitrogen 2·83, 2·66 mg. per cc.; and the L_+ dose 0·022, 0·018 cc. These figures correspond to concentration of binding units per mg. of nitrogen of 33, 43 times and yields of 81, 89 per cent. respectively.

Apparatus.

The description of various ways in which these bags have been mounted for use in the experiments described in this and in the following paper may be of interest.

The simple mounting of one collodion bag shown in Fig. 1 will be found satisfactory for either filtration or pressure dialysis as long as care is taken to make the apparatus really air-tight, or else to attend to it every few hours. The wide glass tube *b* acts as reservoir for some of the fluid which will ultimately replace that forced through the bag, and also for air under pressure. If an auxiliary air vessel fitted with valve and gauge be attached to *a*, instead of the valve and gauge only, the apparatus can be left unattended for days and maintain the desired pressure. In fact, I have found it convenient to have several such vessels ready fitted for use when required: one of them is shown in Fig. 3 (p. 290). By means of a branched tube it can, of course,

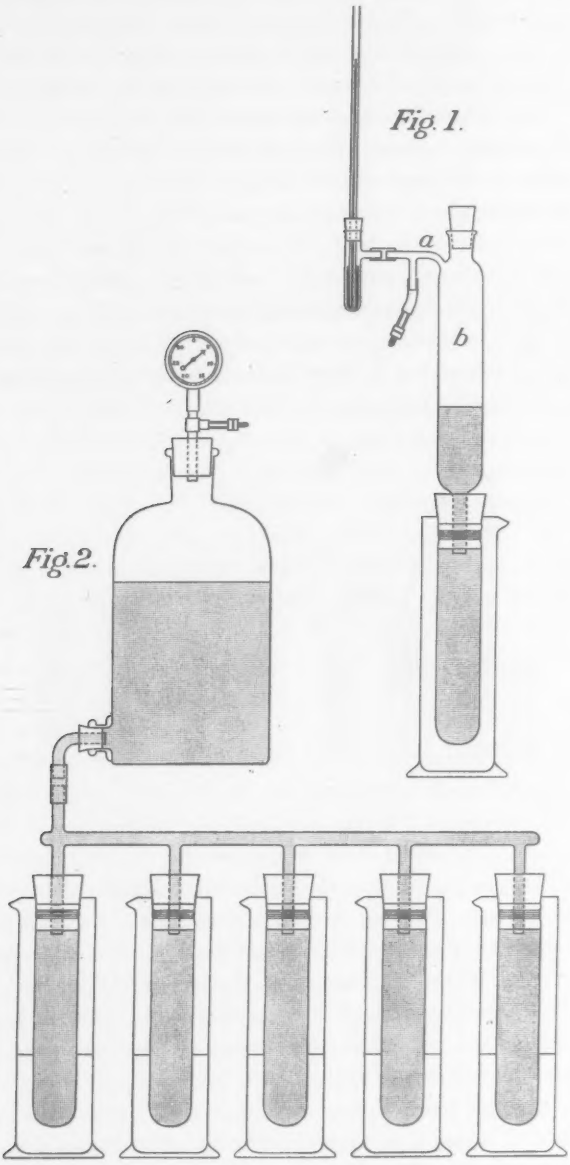


Fig. 1.

Fig. 2.

supply several pieces of apparatus like that sketched in Fig. 1 with air at the same pressure. A convenient mounting for five such bags all holding the same solution is shown in Fig. 2—they are all fed at the same pressure and the filtrates may be compared and so differences in bags investigated. When used for pressure dialysis the water in the jars must be changed from time to time. The inconvenience of not being able to get at the contents of the bags without dismounting the apparatus naturally suggests a continuous apparatus such as that in Fig. 3 for pressure dialysis. The arrangement whereby the toxin and 0·3 per cent. phenol flow in opposite directions effects a very considerable economy in distilled water. The apparatus needs no attention except daily to fill the aspirator with 0·3 per cent. phenol, pump a little air into the reservoir and run off the yield of pressure-dialysed material at *d*. The bottles indicated on the left are double Winchesters holding 5 litres each.

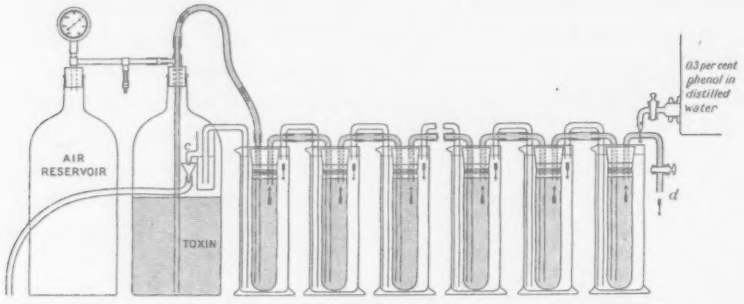


Fig. 3.

The overflow at *c* empties direct to the drains. In two sets of this kind the process has gone on for upwards of three months, in each case without any signs of the bags becoming choked. Apart from the economy of bags, for a set of eight have handled 100 litres of toxin and are still in use, there is possibly the additional advantage that the loss due to adsorption in the bag is minimal, since the volume of toxin handled by each bag is so great. Further details of the working of this apparatus are given in the following paper [Glenny and Walpole, 1915, p. 301].

To avoid the clumsiness of large scale operations in glass apparatus, and the limitation of pressure to that which unsupported bags will stand, it is an obvious step to transfer these operations to properly designed metal

apparatus in which use is made of flat membranes. These, unlike the collodion bags described, are uniform all over and possess the advantages of being capable of accurate calibration and reproduction.

The preparation of flat collodion sheets. Although in practical concentrations a great deal can be done without the use of flat membranes, for a bag can always be made on the "safe" side with a corresponding loss of rapidity of filtration and other properties of more or less value, nevertheless the latter have many advantages. For large scale technical operations impregnated cloths offer greater permanence and strength, but for research work and technical operations on the smaller scale, where there is opportunity to use a reasonable amount of care with the resulting films, I have taken advantage of the increased simplicity of films of water and nitrocotton only. The absence of supporting material such as cloth or canvas makes it much easier to study the properties of these systems accurately, to reproduce them at will and to arrive at a nice discrimination as to the most suitable membrane for any particular purpose. If a thin membrane of high porosity and therefore low tensile strength be advisable for a particular filtration, I suggest that a fine nickel wire gauze or a cloth to support it should be placed not in the membrane but behind it. The homogeneity of the membrane is then not disturbed and measurements such as thickness, tensile strength, permeability to water, water content, etc., bear results which are more intimately applicable to a rational study of the problem in hand.

A square or round sheet of plate glass larger than the finished filter required is levelled accurately, and on to it is poured a measured quantity of "stock" solution suitably diluted with a mixture of equal parts of dry alcohol and ether. Care should be taken that the collodion solution is poured on to the centre of the glass, and that it is sufficiently diluted to run right to the edge. As it will not run over unless an unnecessarily large volume be taken, the glass really acts as a very shallow dish. As evaporation proceeds, the film slowly hardens until it is decided to transfer the glass plate with its attached film to water, beneath which they are separated after the lapse of a few minutes by one steady pull. At any time later a filter the exact size required is cut out of the film with a template and a knife. A good commercial half-inch plate glass is cheap and quite flat enough to give films, say eight inches across, differing between their thickest and thinnest parts by only 40 μ or less. To obtain such films I have taken pieces of glass 12 inches square. Square films will be found thinner at the corners than at the centres of the sides if the volume of collodion poured be insufficient.

A tendency which sometimes occurs to curl up and leave the glass in dry or warm weather may be prevented by painting the edges of the hardening film with "Collodion Flexible B.P."

Now the degree and temperature of nitration determine the physical properties of nitrated cotton to a most marked extent, so that the properties of the film ultimately obtained depend upon a host of factors, of varying degrees of importance, starting with the manner of nitration of the cotton and ending with the pressure applied to it in use. To characterise membranes therefore so that they may be reproduced and their individual properties discussed, the only possible plan is to take in every case a quantitative account of the result of all variables in procedure which are recognised and utilised as such, and by using stock solutions either identical or as similar as possible, and constant conditions, to reduce the other factors as far as is experimentally convenient to constancy.

The variables I have utilised are two in number and both of them are readily controlled and expressed numerically. The first is the weight of dry nitrocotton per sq. cm. (m) of the finished film. This is determined beforehand, for the strength and volume of the solution are known as is also the area of the plate over which it is poured. The second is the "wetness" of the resulting film, a property, intimately connected with its porosity, which depends primarily upon the amount of solvent still remaining in the nitrocotton on the glass plate before it is plunged into water. As a numerical expression of the "wetness" (w) of a filter I have found it convenient to use the ratio of its weight to that of the dry nitrocotton it contains.

For example, 157 cc. of a 3·5 times diluted "stock" solution of Schering's celloidin (13·5 per cent. by weight) were poured on to a circular plate 30·5 cm. diameter.

Hence $$m = 0{\cdot}0082 \text{ g.}$$

The weight of a circular filter 23·5 cm. across cut out of this sheet was 17·5 g. The weight of dry nitrocotton it contains is therefore $\frac{0{\cdot}0082 \times \pi \times 23{\cdot}5^2}{4} = 3{\cdot}56$ g.

Hence $$w = \frac{17{\cdot}5}{3{\cdot}56} = 4{\cdot}91.$$

Direct determinations, by weighing, on small pieces from the remainder of the film after the circular filter had been cut out gave

$$m = 0{\cdot}0081 \text{ g.}$$
$$w = 5{\cdot}02.$$

Now, to test the uniformity of a film it is often convenient to make a few measurements of thickness (t) with a micrometer. The formula connecting m, t and w is found, in practice, to be that which follows at once from the assumption that the thickness of the film is the sum of the thicknesses of the nitrocotton and water in it. The density of nitrocotton may be taken as 2.

$$t = m\left(w - 1 + \frac{1}{\Delta}\right) = m\,(w - 0{\cdot}50).$$

Hence, when making filters to specification it is more convenient not to use the balance at all, but to use a micrometer not only for testing the uniformity of the finished filter, but also for the purpose of determining the correct drying time for any particular film. A thin strip from any part of the film which is just clear of that part which will be required for the finished filter is cut on the glass, peeled off, plunged into water and examined with a micrometer a few minutes later. The thickness gives at once information as to how far the drying has progressed.

Two experimental films, the drying of which was followed in this way, furnished the examples given below:

Example 1. A 13·5 per cent. solution by weight of Schering's celloidin had been diluted 4·5 times. Of this 150 cc. were poured on to a plate 30·5 cm. square, and strips were cut from time to time. Each was immediately after removal from the glass plunged into water, and its thickness measured as the mean of five readings at equidistant points on that part of the strip which would be included in a filter 20·5 cm. square. Direct determinations of m and w were made by weighing pieces of known area both wet and after drying to constant weight. The temperature of the room was 10°–12°.

Example 2. A film containing more nitrocotton per sq. cm. was made by pouring 200 cc. of the "stock" solution above after 3·5 times dilution on to a square plate of the same size. Temperature, 12°–14°.

	Hours of drying before strip was pulled off plate	Mean thickness, t, after immersion in water cm.	Maximum variation from mean thickness μ	m by weighing pieces of known area dried to constant weight	w by weighing pieces wet and after drying to constant weight	w calculated from formula $t = m \times (w - 0{\cdot}5)$
Example 1	2	0·0435	−5	0·00488	9·28	9·41
	3	0·0322	+3	0·00493	6·97	7·03
	4	0·0182	−12	0·00515	4·00	4·03
	5	0·0110	0	0·00503	2·63	2·68
	6	0·0094	−4	0·00512	2·29	2·33
Example 2	2·25	0·0587	+17	—	7·16	—
	3	0·0484	+16	0·00717	7·29	7·25
	4	0·0337	+13	0·00679	5·48	5·46
	5	0·0194	+13	0·00635	3·56	3·55
	6	0·0117	+3	0·00684	2·15	2·21

Press for the use of flat sheets. Fortunately these collodion-water membranes make excellent joints, so that the construction of a press (Fig. 4) to take them without leaking is a comparatively easy matter, and as there is no precipitate to be dealt with, the frames may be thin and narrow and the channels small. Instead of horizontal channels leading from frame to frame I have drilled the frames and soldered in nipples over which suitable rubber tube may be passed. Such a connection between any one nipple and another is instantly made and can be relied upon to stand with certainty for an indefinite period pressures higher than those which I have found it convenient to use. A press permits the employment of pressures which an unarmoured bag could not be expected to withstand, and, constructed in this way, is without prejudice as to the use to which it may be put—whether a periodic or a continuous process—whether filtration, dialysis, or a combination of the two. This is advisable, for such a press as this may well be called upon to do the routine concentration of diphtheria toxin (a continuous pressure dialysis process), that of tetanus toxin (a periodic process—ultrafiltration followed by pressure dialysis), to free tuberculin from glycerol on occasion, and to do any experiments of a similar nature that may be required.

No plates are used in the press—between each pair of membranes and the next on either side are inserted perforated and corrugated material when the press is made up. The celluloid sheet used for keeping apart the lead plates of certain types of accumulators is eminently suitable for the purpose when only low pressures are used. Care must, of course, be taken in filling the press to see that the membranes are not burst by applying pressure before it is full. Although I have done without them in this apparatus, plates with a flat edge but embossed elsewhere with a suitable pyramid pattern would undoubtedly give the advantage of additional security in this respect.

In another apparatus, not illustrated, which I have used as an ultrafilter only, one such plate, ten inches across, covered on each side with a membrane and placed between two flat half-inch steel plates has been used with success. Liquid to be filtered is forced through fine passages in both outer plates and the filtrate runs out from a small hole in the side of that between. The filtering area is 831 sq. cm. and the amount of fluid lost in the apparatus is 2–3 cc., e.g. 50 cc. of blood serum at 2 atmospheres gave in 20 minutes 40 cc. of protein-free filtrate containing sugar and salts but giving no biuret reaction. The residue recovered on gently aspirating at the inlet tubes was 7·5 cc. The filters used were toxin proof, but for the particular experiment quoted much softer and more porous films would have done.

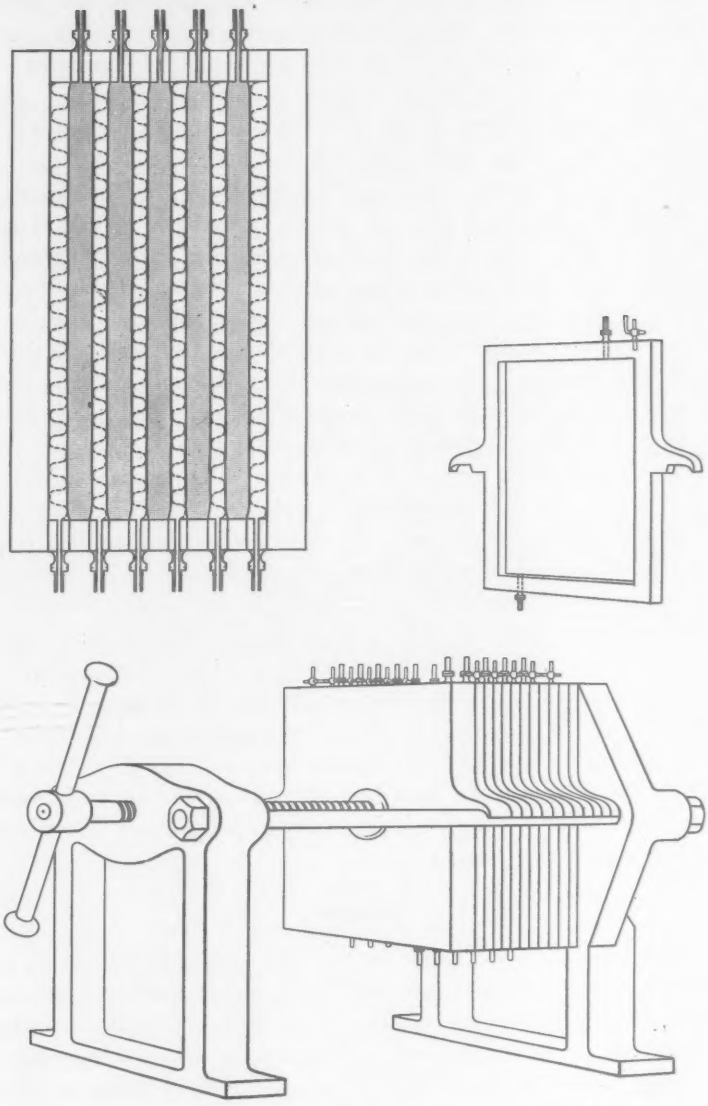


Fig. 4.

The advantages of working in this fashion with apparatus more suitably designed for the purpose than is possible using glass tubes and rubber stoppers only, are so obvious that this point need not be further emphasised.

General remarks. Although requiring a certain amount of special apparatus to support them so that no liquid to be filtered can escape or get round the film into the filtrate, the flat films offer many advantages over those made in test tubes. They are perfectly uniform, may be characterised quantitatively, and are readily and exactly reproducible at will. Dealing with them involves less trouble, for when a flat film is poured the 200 cc. or so of dilute solution is finished with: but when a bag is made the droppings of thick solution from the inverted tube must be collected again in the bottle, and, on account of evaporation, the composition of the "stock solution" is continually changing. I have examined strips of flat films in a paper testing machine and plotted stress-strain diagrams of them. Their liquid nature, for they continue to stretch at almost constant tension, is, as one would anticipate, particularly marked in those whose main constituent is water. The tensile strength increases remarkably rapidly as "wetness" falls for films of the same nitrocotton content. There is every indication that impermeability to large molecules does the same thing. Unfortunately, experiments designed to test whether tensile strength could be correlated in the same way with m and the average size of the pores could not be continued: though an investigation on these lines could not fail to give instructive results.

In conclusion I would urge that any investigation of porosities of films to proteins should be accompanied by determinations of p_{H}., and a statement of the electrolyte content of the materials examined, and the nature of the preservative, if any, used. It is now fully recognised how fundamentally these conditions affect the state of aggregation of proteins, and the adsorption of other materials by them.

Summary.

Some notes have been made on test tube shaped bags prepared from collodion, and used for simple ultrafiltration and dialysis experiments. Their lack of uniformity and the difficulty of accurate reproduction militate against their use for careful work. Their circular section is a further disadvantage in that the area of the film operative per unit volume of material treated is large only when the tubes are small, and that, unless the bags be armoured, the pressure that can be used is limited to that which the unsupported membrane can withstand.

Flat membranes may be made by pouring alcohol-ether solutions of collodion on to levelled plate glass, and then plunging the film, from which alcohol and ether are more or less removed by evaporation, into water. Using this method, and taking certain precautions described, they may be made with remarkable uniformity. By keeping as far as possible to constant conditions, these membranes may be described fully, for purposes of reference and reproduction and as a guide to their properties, by stating the kind of nitrocotton used, the weight of it per sq. cm. they contain, and the "wetness," i.e. the ratio of the weight of a piece of film to its weight when dried to constancy. Films, made in the manner described, containing 8 mg. per sq. cm. of the particular nitrocotton used and weighing, with contained water, 40 mg. per sq. cm., or others where $m = 5$ and $w = 17{\cdot}5$, have been found to retain quantitatively all antigens with which experiments have been made. Practical applications of this result are given in the paper following this [Glenny and Walpole, 1915]. Their permeability to simpler molecules, salts, and water remains high, and filtration through them is rapid.

It is suggested that in these films, more than in other ultrafilters in general use, the channels leading through the gel structure from one side to the other are of a certain uniformity in size. None of them is large enough to allow any antigen to pass; but the structure is highly porous and, because there are many such channels, rapid ultrafiltration results.

Arrangements of apparatus are described whereby films of this type whether flat or in the shape of test tubes may be made and utilised for ultrafiltration and pressure dialysis.

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XXVII. DETECTION AND CONCENTRATION OF ANTIGENS BY ULTRAFILTRATION, PRESSURE DIALYSIS, ETC. WITH SPECIAL REFERENCE TO DIPHTHERIA AND TETANUS TOXINS.

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One has but to know something of the precarious character of routine toxin production to be aware that the artificial conversion of low value toxins to those of high titre is a problem of more than mere academic interest.

In the search for a suitable process by which this may be done it is soon realised that purely chemical methods such as those referred to so completely by Pick [1912] are not available, as they are usually cumbersome and costly, and any effective concentration invariably entails an enormous loss of antigen. Amongst other possibilities the ammonium sulphate method of Heinemann [1908] failed in my hands, possibly because phenol was present; while an alcohol method, applied by Banzhaf, I have not tried on account of expense, for it is essential that any routine process should be simple and cheap. The matter was left at this stage for several years until the accidental discovery was made that on acidification diphtheria toxin, from which salts had been removed by dialysis in a collodion membrane, developed a slight precipitate which contained all the toxin. Moreover, collodion membranes of a special kind [Walpole, 1915] could be made which, though reasonably permeable to water, salts, etc., nevertheless retained quantitatively diphtheria toxin and

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all other antigens with which experiments were subsequently tried. These unexpected results, in flat contradiction to those of Baroni [1911] for example, who found diphtheria and tetanus toxins to pass through the collodion membranes he employed, form the basis of the concentration methods here put forward. The various experimental difficulties have disappeared as the result of experience, but, so far, it is only in the case of diphtheria toxin that precipitation by a trace of acid after the removal of the salt can be used as an effective short cut to a purification of the antigen. The invariable result, so far as my experience goes, that antigens do not pass through these membranes suggests their employment not only for routine toxin purification, but in addition as a laboratory test to form a first opinion on the specificity or otherwise of the toxic constituents of any cultural fluid.

The Purification of Diphtheria Toxin.

A batch of diphtheria toxin is appraised by the immunologist by its "binding unit" content per cc., a quantity which for all purposes is most readily measured as the L_+ dose, though this would not necessarily be so if the original media were not similar or had not disintegrated, during the growth of the bacillus, on similar lines. Also, the reservation is made that the "binding units per cc." of these broths are by no means *proportional* to their utility: rather some such scale as the following is taken:

L_+ dose	Use for immunising
0·15 cc.	"excellent"
0·30 cc.	"good"
0·50 cc. and over	"unusable."

In other words poor toxin cannot be made to act as good toxin simply by taking more of it, for experience has shown that the development of antitoxin in the horse is greater when the effective immunising material is interfered with in its action as little as possible by accompanying nitrogenous matter.

It may therefore be taken as a working hypothesis, remembering that the experience of the immunologist is the final and only appeal, that, providing the loss of toxin is minimal, the extent to which the binding units per milligram of nitrogen is increased by this purification process is some sort of measure of its efficiency. When the material accompanying the toxin is so far reduced as to be physiologically of no importance in the dose accompanying the greatest toxin injection given, it is obviously not a true measure because

further purification serves no useful purpose: it may be inadvisable, in fact, for one would suspect, on the ground of general experience, an increased instability of the toxin as it approaches purity.

The essential process we have employed to concentrate and purify diphtheria toxin is to dialyse it under pressure using a collodion membrane of a particular kind dealt with more fully, for the sake of convenience, in a separate paper [Walpole, 1915]. The dialysed material is then acidified and centrifuged. The precipitate, dissolved in a trace of alkali, constitutes the concentrated material. It should be kept in the cold after the addition of a small quantity, say, 0·3 per cent., of phenol.

To know when the material has dialysed sufficiently to pass on to acidification either of the following methods may be used. A sample may be tested by adding 0·3 cc. N acetic acid to 10 cc. If the precipitate formed flocculates well and leaves a bright supernatant fluid no further dialysis is necessary. This point corresponds, for the broths which have been dealt with, to that where no more colouring matter passes out through the walls of the bag and to a conductivity of about 0·00078, i.e. that of 0·0065 N KCl. For routine work with a continuous apparatus [Walpole, 1915, p. 290] rough conductivity determinations are much the most convenient method for checking the working and determining how much dialysed toxin may be drawn off each day in safety.

Results.

Where this process is applied the cost of toxin production is considerably reduced, for batches are always worth the units they contain, whether they are good or bad, and no toxin however poor need be thrown away. Periodic recurrences of bad toxin to which every laboratory is subjected need, therefore, no longer be feared.

The following generalisations summarise the results of our experience upon which this concentration process for diphtheria toxin had been developed.

1. *Whether by pressure dialysis or ultrafiltration no toxin passes through these membranes.*

These results have been checked so many times that special experiments need not be cited. Part of the evidence supporting them will be found in the ensuing pages. [See also Walpole, 1915, p. 288.]

As a matter of convenience, however, the details of some routine concentrations are given at this juncture, for they depend essentially upon the impermeability of these bags to diphtheria toxin.

At the time of writing two sets of apparatus [Walpole, 1915, Fig. 3] each consisting of eight bags which were set up some months ago are still in use. One has handled over 100 litres; the other about 44 litres, of which two small batches were worked out more or less completely.

Details of concentration experiments. March 12th to April 1st. Using the continuous apparatus [Walpole, 1915, Fig. 3], 12 litres of diphtheria bouillon, J 2520 (L_+ dose = 0·40 cc.: nitrogen = 2·8 mg. per cc.) gave, in 20 days, 8500 cc. of pressure dialysed material (nitrogen = 1·96 mg. per cc.). The process was not hurried in any way; nothing was drawn off for three days, then 500 cc. per day were collected through the run, and then a couple of days allowed to elapse before all the bags were emptied. The excess of pressure used was 0·3 atmosphere and the consumption of the dialysing fluid, 0·30 per cent. phenol in distilled water, ten litres per day. After acidification the precipitate, representing twelve litres of the original bouillon, was dissolved in alkali and diluted to 250 cc., giving a solution whose L_+ dose was found to be 0·012 cc., and the nitrogen per cc. 2·80 mg.

The result may be roughly stated that, with a loss of 30 per cent., the binding units per mg. nitrogen and also the binding units per cc., have been increased 33 times.

The previous batch of toxin treated by this set of collodion bags was J 2530, of which 11·5 litres (L_+ dose 0·40 cc.: nitrogen 4·6 mg. per cc.) gave 230 cc. of concentrated material (L_+ dose 0·01 cc.: nitrogen 6 mg. per cc.) representing a yield of 80 per cent.

When the same set of bags had handled 50 litres of material whose L_+ dose was 0·4 cc., a sample of dialysate from *C* was collected. They were then lifted clear from the glass jars containing them and some of the ultrafiltrate dripping from them collected (that collected in the first hour or two was, of course, discarded). Neither of these solutions interfered with the increase of weight of the guinea pig when injected in a dose of 1 cc.

The lost toxin, therefore, does not pass through the bags, nor is it to be found in the supernatant fluid above the precipitate produced by acidification. It would seem probable that it represents destruction by light, or by being left at room temperature and in a state of increased purity at a reaction which is probably not that at which it is most stable, at some stage in the process. Experiments on this point are in progress. Should the loss be actually in the filter itself the recovery therefrom of a highly toxic material may be anticipated.

2. *The toxin goes through the best and thickest parchment commercially obtainable, though the parchment is far less permeable to water than the collodion membrane through which toxin cannot pass.*

A large volume of toxin J 2536 (L_+ dose = 0·35 cc.) filtered through parchment under a slight pressure yielded after some days 50 cc. of filtrate resembling the original in colour and general precipitation reactions. The L_+ dose of the filtrate was 0·60 cc. This is a striking example of the fact that low permeability of a membrane to water is not necessarily accompanied by low permeability to toxin. Using the same batch of toxin and a collodion membrane ($m = 5$: $w = 3·5$, [see Walpole, 1915]) no toxin could be detected in the filtrate (1 cc. nil), though the volume of the filtrate per square centimetre per hour was many times greater in this case. Below are given details of a repetition of this experiment, using another sample of the parchment and another collodion bag.

Collodion bag.	Parchment bag.
Area exposed 260 sq. cm. Time 24 hours. Filtrate 8 cc., of which 1 cc. has no effect on the guinea pig. Pressure 6″ water.	Area exposed 1960 sq. cm. Time 24 hours. Filtrate 6 cc. of which 0·1 cc. kills a guinea pig. Pressure 7″ water.

This point is, in our opinion, worth full and patient investigation because of its far-reaching consequences in bacteriological work generally.

3. *The amount of toxin in a cultural filtrate bears no relation to the amount of precipitate obtained on acidification after pressure dialysis through these membranes.*

4. *The method is universally applicable at least to all brews of diphtheria toxin of the type made in these laboratories.*

Samples of 200 cc. each from twenty-nine different brews of toxin were dialysed, each in a separate bag, under pressure for four days against distilled water changed daily. Colouring matter had then ceased to come out through the bags and the specific conductivity of the inside fluid was in every case less than 78×10^{-5}. All gave nicely flocculated precipitates on adding 6 cc. of 1·0 N acetic acid to the contents of each bag. They were placed in order of the amounts of their precipitates as well as could be judged by eye. The first fifteen precipitates were then mixed and dissolved in 60 cc. of distilled water. Equal volumes of the corresponding original brews were also mixed. Both solutions were tested for "binding units" with the following results.

The first fifteen samples amounted in volume to 3000 cc. (L_+ dose = 0·45), from which were obtained 60 cc. of concentrated material (L_+ dose = 0·012).

The results referring to the remaining fourteen samples which all gave smaller precipitates when treated in the same way may be summarised:

2800 cc. (L_+ dose = 0·45) gave 56 cc. (L_+ dose = 0·012).

Though no other attempts were made solely to settle these points they are confirmed by the general experience of numerous other concentrations. The yield in both the above cases is 80 per cent., and the concentrations of the binding units per unit volume fifty times.

5. *If the cultural filtrate after pressure dialysis is only partially precipitated by a trace of acid, and if, after removing this small precipitate, it is completely freed from precipitable matter by further acidification, then nearly all the toxin will be found in the first precipitate.*

Four litres of a mixture of toxins (L_+ dose = 0·40: nitrogen 4·63 mg. per cc.) gave as first fraction material which, dissolved in a trace of alkali, was diluted to 80 cc. The L_+ dose was 0·008 cc. and the nitrogen content 3·5 mg. per cc. in this solution. The second fraction, comprising the rest of the precipitable material, when similarly treated gave 80 cc. of a solution of L_+ dose 0·30 cc. and nitrogen content 6·02 mg. per cc. Hence the yield of binding units on the first precipitate is as near as one can tell quantitative, and that on the second, which contains nearly twice as much nitrogen, is only 2 per cent. of the total binding units originally taken.

This experiment was performed either to confirm or throw doubt upon a previous result in which the distinction between the binding unit content per mg. nitrogen of successive fractions was equally marked.

6. *There is no evidence that this routine concentration process brings about any separation of the toxic from the binding elements of the cultural filtrate.*

The following two experiments were made with a batch of toxin J 2538, the L_+ dose and M.L.D. of which were 0·38 cc. and 0·005 cc. respectively. In both of them the concentrations of L_+ dose and M.L.D. were, within the experimental error, equal.

Experiment 1. 200 cc. of original toxin yielded a trace of precipitate which was dissolved in a convenient volume, which amounted to 16·6 cc. The L_+ dose of this solution was 0·05 cc. and M.L.D. 0·0007 cc. It is seen that both the toxicity and the binding units have been concentrated to about the same extent, i.e. 7·1 times in the one case and 7·6 times in the other.

Experiment 2. 1800 cc. of original toxin yielded 28 cc. of concentrated toxin having L_+ dose 0·008 cc. and M.L.D. 0·0001 cc. The concentrations are, in this case, forty-eight times for the L_+ dose and fifty times for the toxicity.

7. *Some change occurs in the material undergoing pressure dialysis which is not reversible, for it is impossible to reconstruct the original material by adding to the fluid remaining in the bag a corresponding amount of dialysate after concentration to the correct volume.*

The original material gives no precipitate on acidification: the reconstructed material does, i.e. it appears as if the salt when once removed fails to inhibit precipitation by acid when again added.

500 cc. of diphtheria toxin, brew Y 76, gave 5 litres of pressure dialysate (A) against 0·3 per cent. phenol, with a reduction in volume to 180 cc. (B). Comparative tests showed that the addition of 2 per cent. sodium chloride reduced by only very little the precipitate obtained by acidification. Similarly, if 170 cc. of A concentrated ten times by evaporation be added to 9 cc. of B, almost a full precipitation still occurs.

The immunising value of concentrated diphtheria toxin.

In the light of experience it is easy to establish whether any particular toxin has good immunising value or not, but to demonstrate statistically the comparative values of two toxins would require many horses owing to their individual variations.

Two normal horses immunised with concentrated toxin prepared from material so poor in value as to be unusable reached 800 A.T. units and 1400 A.T. units per cc. Individual horses immunised with unconcentrated toxin have reached 2000 A.T. units per cc., but it requires more than usually good toxin to reach an antitoxic unit content of even 1400 per cc. The average value of horses under similar treatment with good unconcentrated toxin was 700 units per cc.

The details of the response to treatment, although referring to two horses only, indicate clearly that concentrated toxin is at least as good as unconcentrated toxin unit for unit for purposes of immunisation. The volume of the injections was very small, though the number of binding units injected was the same as that usually given when unconcentrated toxin was used. The general condition of both the horses was far better than that of horses immunised in the ordinary way.

Another horse that had received several immunisations of unconcentrated toxin was gradually falling off in unit value. After a course of concentrated toxin, in which the largest dose given was 10 cc., higher value sera were obtained from it than had been obtained at any time during the previous treatment.

These experiments on the immunising value of concentrated diphtheria toxin, as compared with that not so concentrated, are being continued.

Purification of Tetanus Toxin.

Owing to the vigorous digestion of the broth constituents by the tetanus organism the concentration of toxin per mg. nitrogen obtained by ultrafiltration followed by pressure dialysis is in this case greater even than in that of diphtheria cultural filtrates. The rapidity of filtration is, too, for the same reason, much greater. The slight precipitate obtained on acidifying the dialysed material is, as has been already stated, not toxic. This curious difference between diphtheria and tetanus toxins must be of considerable theoretical significance.

In the tests given below the animal used was the mouse.

Experiment 1. An experiment has been described [Walpole, 1915, p. 288] where comparative tests of the porosities of two bags showed that no toxin could be detected in one of the filtrates and very little in the other. A dose of 0·0005 cc. of the original material placed in the bag caused death on the 4th day.

Experiment 2. Toxin BDC (0·0005 cc. death on 4th day); 30 cc. were concentrated to one-seventh the volume with continual change of water outside, and diluted again to its original volume: no change in M.L.D. could be detected. A small precipitate obtained by acidifying and centrifuging an aliquot portion was found to be non-toxic.

Experiment 3. 190 cc. of toxin BDC were reduced to 6 cc. as above. Of this

0·00005 cc. gave death on the 3rd day
0·00002 cc. „ „ 7th „

that is to say, that the toxin was concentrated about twenty times and the volume reduced to one-thirtieth.

Experiment 4. Toxin BDA (L_+ dose 0·05 cc.); 10 cc. of this were reduced to about 3 cc. and then made up to the original volume with physiological saline solution. The L_+ dose was found to be unaltered.

Experiment 5. 10 litres toxin BKA (1 cc. contains 3·7 mg. nitrogen) gave in the apparatus described by Walpole [1915, Fig. 2] in six days 9630 cc. of ultrafiltrate divided between five bags. Tests on these filtrates showed that one bag only allowed a detectable amount of toxin to pass through (M.L.D. 0·4 cc.). Twenty-four hours' pressure dialysis followed, and then

300 cc. of concentrated and purified toxin containing 3 mg. of nitrogen per cc. were collected from inside the bags.

It would seem from these observations that the horse, while still receiving the full measure of specific antigen, may be spared 97 per cent. of the nitrogen and, in this case all the indole, fatty acids, etc., of a particularly noxious and evil-smelling fluid.

As in the case of diphtheria toxin the cost and labour of the production of the tetanus antigen may be reduced by this means, for the value for immunising purposes of a given toxin is that of the number of units it contains. Also, a greater certainty in immunising horses and again a saving in the cost of horses and their keep during immunisation may be confidently anticipated.

Experiments with other Cultural Filtrates.

It has already been suggested that one of these membranes might be used to form a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid.

A toxic filtrate from a culture of the malignant oedema bacillus (Besson) grown in these laboratories by Mr Buxton was found to give an ultrafiltrate of undiminished toxicity. This observation indicated the probability that the toxic substance was not of the nature of a specific antigen. The filtrate in question was further investigated by Barger and Dale, who found that its essential toxicity was due to the presence of a high concentration of ammonia[1].

On the other hand, the toxic constituent of a broth culture of an organism producing a fatal epidemic in the squirrel was found to be retained by one of these bags. The suspicion that this would be a specific antigen was confirmed by Mr Buxton, who is investigating the outbreak, for he was able, using the toxin in its concentrated and purified state, to produce a corresponding antitoxin in the goat. Pressure dialysis applied to tuberculin gave in the two cases tried a complete removal of the glycerol without detectable loss of antigen. Mr Buxton, to whom I am again indebted for animal tests, employed Koch's method, using sensitised guinea pigs. For ophthalmic tests where the presence of glycerol is not permissible, or for low potency tuberculins where further concentration by evaporation is impossible, the method should prove to be of considerable utility in view of the waste and inconvenience of the alcohol precipitation method at present practised.

Mallein has also been concentrated and freed from glycerol in the same way.

[1] Private communication by Drs Barger and Dale.

Filtration of pituitary active principle, etc.

Although they are not antigens, the behaviour of secretin and the active principle of pituitary extract when filtered under pressure through these bags is of considerable interest. A simple pituitary extract gave a filtrate which, compared with the original solution and the residue, showed that although much of the active principle had passed through it had not done so at the same rate as the water, salts, etc. A similar result was obtained with the co-enzyme of zymase, in an experiment in conjunction with Prof. Harden, to whom I am indebted for the material and the tests.

Dr P. P. Laidlaw has communicated to me privately that one of his secretin preparations, filtered through a standard bag into saturated picric acid, gave at once a crystalline precipitate of marked activity.

I wish to express my indebtedness to the assistance of Mr Ralph Defries during the course of this work.

Summary.

Experiments are described in which the permeability of collodion-water membranes of a special type to various antigens was investigated. Owing to the fact that none of the antigens tried was found to pass through these membranes their use is suggested for the purpose of forming a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid. The recognition and subsequent concentration of the antigen of a fatal epidemic in the squirrel was effected by this means, and an investigation of the nature of the toxicity of filtrates of malignant oedema cultures led to its complete elucidation.

Mallein and tuberculin were freed from glycerol and a considerable quantity of nitrogenous material in this way.

A process for the concentration and purification of diphtheria toxin which at a yield of 70–80 per cent. diminishes the nitrogen content about 50 times has been fully worked out. It is performed by dialysis under pressure, followed by acidification, centrifugalisation, and re-solution of the small precipitate obtained in a trace of alkali.

If a continual supply of toxin having L_+ dose 0·20 could be relied upon, this artificial conversion of low value toxin to that of high titre, would remain more of academic than practical interest. But, the majority of batches do not reach this high level and hence this concentration

process is of considerable importance. One thing is certain and that is that the periodic recurrence of "bad" toxin to which every laboratory is subjected need now no longer be feared.

By ultrafiltration followed by pressure dialysis a concentration of tetanus toxin may be effected which should be of considerable practical value, for the disintegration products of the broth materials which accompany the antigen are particularly noxious in this case.

These membranes are impermeable to enzymes, but allow to pass secretin, the pituitary active principle, the co-enzyme of zymase, and the toxic constituent of Witte peptone.

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